OCCURRENCE OF VANCOMYCIN RESISTANT ENTEROCOCCI (VRE) IN PIGS, PORK AND THEIR ENVIRONMENT

Thesis

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Veterinary Science
(Veterinary Public Health and Epidemiology)

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Dedicated to....

My Beloved Father

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ABBREVIATIONS

°C	:	Degree centigrade
%	:	Percent
µL	:	Microliter
AP	:	Andhra Pradesh
Bp	:	Base pair
BEA	:	Bile esculin azide agar
BHI	:	Brain Heart Infusion
BPW	:	Buffered peptone Water
cm	:	Centimetre
^{0}C	:	Degree Celsius
gm	:	Gram
gm/l	:	Gram per litre
H_2S	:	Hydrogen Sulfide
LB	:	Luria –Bertani
mg	:	Milligram
MIC	:	Minimum Inhibitory Concentration
mg/l	:	Milligram per litre
MHA	:	Muller Hinton Agar
ml	:	Millilitre
mm	:	Millimetre
MR	:	Methyl red
nm	:	Nanometre
NA	:	Nutrient Agar
ODc	:	Optical density Value of control
ODi	:	Optical Density value of isolates
PCR	:	Polymerase Chain Reaction
UP	:	Uttar Pradesh
VP	:	Voges Proskauer
VRE	:	Vancomycin resistant enterococci

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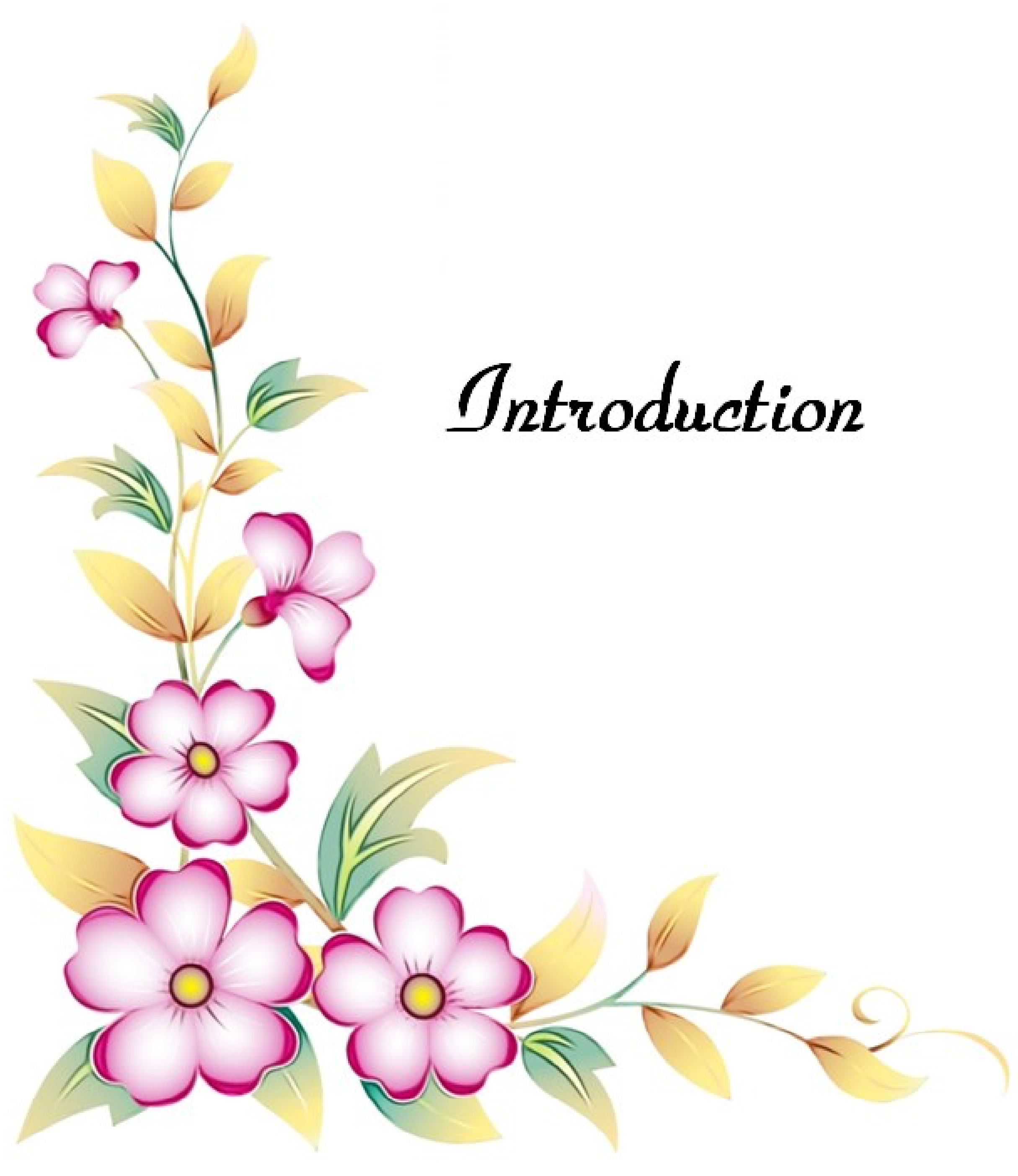
Introduction



1 INTRODUCTION

Microorganisms have a dynamic connection with the biosphere as a result of their constant adaptation to changing environmental conditions, resulting in vast genetic variety. This relationship with the individual can range from beneficial to outright deadly. One particularly interesting common group of inhabitants of this environment is genus of Gram-positive cocci, *Enterococcus* (Biswas, 2015).

Bacteria belonging to *Enterococcus* genus are Gram-positive, catalase-negative, facultative anaerobic and non-spore-forming. Enterococci are commensal bacteria living in the gastrointestinal tract (GI) of many animals, from invertebrates to humans. More than 50 species of *Enterococcus* have been defined, but *Enterococcus faecium* and *Enterococcus faecalis* are the most identified in both humans and animals (Ramos *et al*., 2020). Enterococci will grow between 10^0C and 45^0C temperatures, can tolerate 60^oC for at least 30 minutes and can grow at 9.6 pH and in 6.5 % (w/v) sodium chloride (Sherman, 1937). These bacteria are extremely resilient creatures that can sustain harsh environmental conditions for months at a time, as well as host defences (Selleck *et al*., 2019; Ramos *et al*., 2020). For these reasons, both in people and in animals, enterococci are regarded as opportunistic pathogens and they are often causes of nosocomial infections (Kubasova *et al*., 2017). Until 1984, the enterococci were considered as part of the genus *Streptococcus*, but they have constituted a unique taxonomic entity since the mid-1980s (Nilsson, 2012; Werner *et al*., 2013). Moreover, ecology and epidemiological studies have reported *E. faecalis* and *E. faecium* as frequently isolated from food products like fish, pork, sausages, cheese and minced beef and the environment (sewage, soil, and water) (Torres *et al*., 2018). Due to their preferred intestinal habitat, their

wide occurrence, robustness, and ease of cultivation, enterococci are used as indicators of faecal contamination and are part of the hygiene standards for water and food products. For human and veterinary resistance surveillance systems, they are also appropriate as significant key indicator bacteria. (Werner *et al.*, 2013).

The main problem associated with enterococcal infections is antimicrobial resistance. On one hand enterococci are intrinsically resistant to some antimicrobials; on the other hand, they are very able to acquire and transfer resistant genes from other bacteria via plasmids and/ or transposons (Torres *et al.*, 2018). The selective pressure linked to widespread use of antimicrobials drives an accumulation process of resistance genes in these bacteria and the selection of multidrug-resistant strains. As consequence, antimicrobial therapy of *Enterococcus* infection in humans need to be constantly modulated and changed (Miller *et al.*, 2020). Traditionally, enterococci were regarded as a harmless commensal bacterium, and were even believed to have positive effects on a number of gastrointestinal and systemic conditions. However, when the commensal relationship with the host is disrupted, enterococci can cause invasive infections (Lengfelder *et al.*, 2019).

Enterococci are opportunistic pathogens associated with serious and life-threatening infections to humans such as blood stream infections, infections related to urinary system and endocarditis. The majority of human enterococcal infections are caused by *E. faecalis* and *E. faecium*, which are also a primary cause of hospital-acquired and multidrug-resistant infections (Lebreton *et al.*, 2013).

Enterococcus faecium is classified in a bacterial group that are referred to as 'the **ESKAPE** bacteria'. This is a group of bacteria which escapes lethal action by the antibiotics and associated with healthcare-associated infections in developed and also in developing world. More than 40 percent of infections in ICU patients are caused by the ESKAPE bacteria (Hidron *et al.*, 2008). Antibiotic-resistant *E. faecium* is the utmost common gram-positive bacteria in healthcare-associated infections in the twenty-first century (Arias and Murray, 2008). Also, enterococci can transfer their antibiotic resistance to methicillin resistant *Staphylococcus aureus* (MRSA) leading to even more severe threat (Flannagan *et al.,* 2003). Because of *E. faecium*'s propensity to accept foreign DNA, antibiotic resistance genes accumulated quickly.

Recently, researchers discovered usage of antibiotics such as cephalosporins during antibiotic treatment could create the microbial imbalance in the large intestine thus allowed the outgrowth of multidrug resistant *E. faecium* (Hendrickx *et al*., 2015). Resistance to many of antibiotics, spanning to most of the antimicrobial classes, has increased in *E. faecium* isolates over the previous two to three decades. (Hegstad *et al*., 2010).

Enterococci have gained significance as an important nosocomial pathogen mainly due to their resistance to the commonly used antimicrobial agents such as aminoglycosides, cephalosporins, semisynthetic penicillin (Marothi *et al*., 2005). Multiple antimicrobial drug resistance among the enterococci further compounds the problem of *Enterococcus* infection and presents great therapeutic challenge (Moellering, 1992). Unselective antibiotic use and growth stimulants in animals may be one reason for the arrival of resistant bacteria. The occurrence of multiple drug resistant (MDR) enterococci has been recorded in different kinds of food products of animal origin (Klein *et al*., 1998; Pavia *et al.,* 2000) and there is possibility that these MDR enterococci are transmitted to man by food chain (Bates, 1997). Enterococci have been known for over a century for their potency to cause infections in humans (MacCallum and Hastings,1899; Sherman,1937). Initial reports on enterococci as pathogens have shown that enterococcal infections were limited in numbers and occurrence and mostly caused by the single species, *E. faecalis*. A variety of antimicrobial therapy is being used against enterococcal infections. Ampicillin is used as drug of choice against susceptible enterococcal infections. Emergence of resistance to multiple antibiotics and its capability to survive at elevated levels of these drugs has significantly complicated the management of enterococcal infections. Vancomycins are used under conditions of penicillin resistance and allergy others like linezolid, daptomycin and tigecycline are used under conditions of Vancomycin Resistant *Enterococcus* (VRE) (Mac *et al*., 2003). Epidemiological studies have shown substantial health and also economic consequences from VRE linked infections and persistent colonization in human medicine since the first reports of VRE in 1980s (Leclercq *et al*., 1988). VRE, further, is an uncommon source of illness in animals, and it is rarely found in companion animals (Boehm and Sassoubre, 2014).

In farm animals, *Enterococcus* infections are uncommon (Aphis, 2014) and they are rarely specifically targeted with antibiotics in these settings. However, as a normal part of their commensal intestinal microbiota, *Enterococcus* spp. are exposed to antibiotics administered to animals to prevent or treat infections caused by other bacteria or given in sub-therapeutic doses to achieve the growth-promoting effects (banned in the EU in 2006 and in the US in 2017 and currently allowed in Brazil and China) (Daniel *et al*., 2015; Roth *et al*., 2019; Ibrahim *et al*., 2020). Therefore, the use of antibiotics in food animal production has been associated with the development of antimicrobial resistance (AMR) in enterococci (Hayes *et al*., 2004; Gadde *et al*., 2018). The antimicrobial-resistant bacteria that have emerged and live in the animal production environment are observed to spread to humans via direct or indirect human–animal contact or via the consumption of or contact with animal products (Marshall and Levy, 2011; Daniel *et al*., 2015; Fan and Archbold, 2015). While the *E. faecium* isolated from human samples tend to be of different types than the *E. faecium* isolated from animal samples, the same types of *E. faecalis* isolates have been found in both humans and other animal species. This suggests that the antimicrobial-resistant *Enterococcus* strains may be capable of transmission from animals to humans (Hammerum, 2012). In addition to the possible risk of inter-host transmission, these bacteria harbour a pool of mobile genetic elements and may serve as a reservoir for acquisition of antibiotic resistance genes. Thereby, they could also contribute to the spread of resistance genes by distributing them among Gram-positive bacteria, including the possible transfer of resistance genes from animal-associated *Enterococcus* to human bacteria (Marshall and Levy, 2011; Radhouani *et al*., 2011).

Antibiotic resistance is a global health issue that has been connected to issues involving people, animals, and the environment (George *et al*., 2020). Resistant superbugs can emerge in a variety of habitats due to the persistence and spread of resistant microbial species and the association of factors at the human-animal-environment interface (Aslam *et al*., 2021). The term One Health recognises the importance of increasing interdisciplinary collaboration in human, animal, and environmental healthcare to enable the creation and implementation of programmes, policies, and legislation to improve public health (Founou *et al*., 2016; McEwen and Collignon, 2018). Vancomycin-resistant Enterococcus (VRE) is among the first antibiotic-

resistant bacteria documented whose primary origin is animal farming (Bager *et al*., 1997; Aarestrup, 2000) and there are various studies on the detection of these multiresistant bacteria in swine livestock (Birkegard *et al*., 2019; Xuan *et al*., 2021).

Sakka and co-workers (2008) put forward the hypothesis that VRE bacteraemia was caused by "exogenous" causes such as allogenic bone marrow transplant, chemotherapeutic drug exposure, hypoalbuminemia, and urinary catheter. When it came to vancomycin-sensitive enterococci (VSE), "endogenous" factors such as age, previous GI disease and surgeries related to abdomen were the utmost common causes of pathogenicity. colonization by VRE have been observed to be widespread in patients developing bacteraemia. The increased mortality rate caused by VRE is linked to vancomycin resistance. To limit the occurrence of VRE bacteraemia, clinical environments should be changed, and infection control procedures should be modified and reduce the excessive antibiotic use.

In the US and Europe, the most commonly isolated bacteria from hospital-acquired illnesses are enterococci. Enterococci have gained significance as significant nosocomial pathogen as they are resistance to the commonly and normally used antimicrobials such as aminoglycosides, cephalosporins, semisynthetic penicillin capability to rapidly gain virulence and MDR genes (Kristich *et al*., 2014).

The prime reason for this seems to be the ability of the organism to acquire resistance determinants from related strains and spontaneous mutations within the bacterium (Patterson and Zervos, 1990). High level gentamicin resistance (HLGR) has been a cause of concern in many hospitals associated infections. Between 2003 and 2008, the occurrence of HLGR enterococci increased by tenfold (Rosvoll *et al.*, 2012).

Antibiotics are frequently used as growth boosters in animal farms, which exacerbates the problem. Resistance to vancomycin is either intrinsic or acquired through the possession of nine types of vancomycin resistance genes (*vanA*, *vanB*, *vanC*, *vanD*, *vanE vanG,van L,vanM and vanN)* (Miller *et al*., 2014). *vanA* and *vanB* are the most common genotypes of vancomycin resistant enterococci (VRE) (especially among *E. faecalis* and *E. faecium*) in humans and animals (Miller *et al*., 2014).

Enterococcus species being a common microbiota of livestock including pig, they may be transferred from pigs to humans. The vancomycin resistant *Enterococcus* species in pigs, pork, pig handlers and environment remain understudied in India. Keeping in view the importance of VRE the present study was proposed with following objectives.

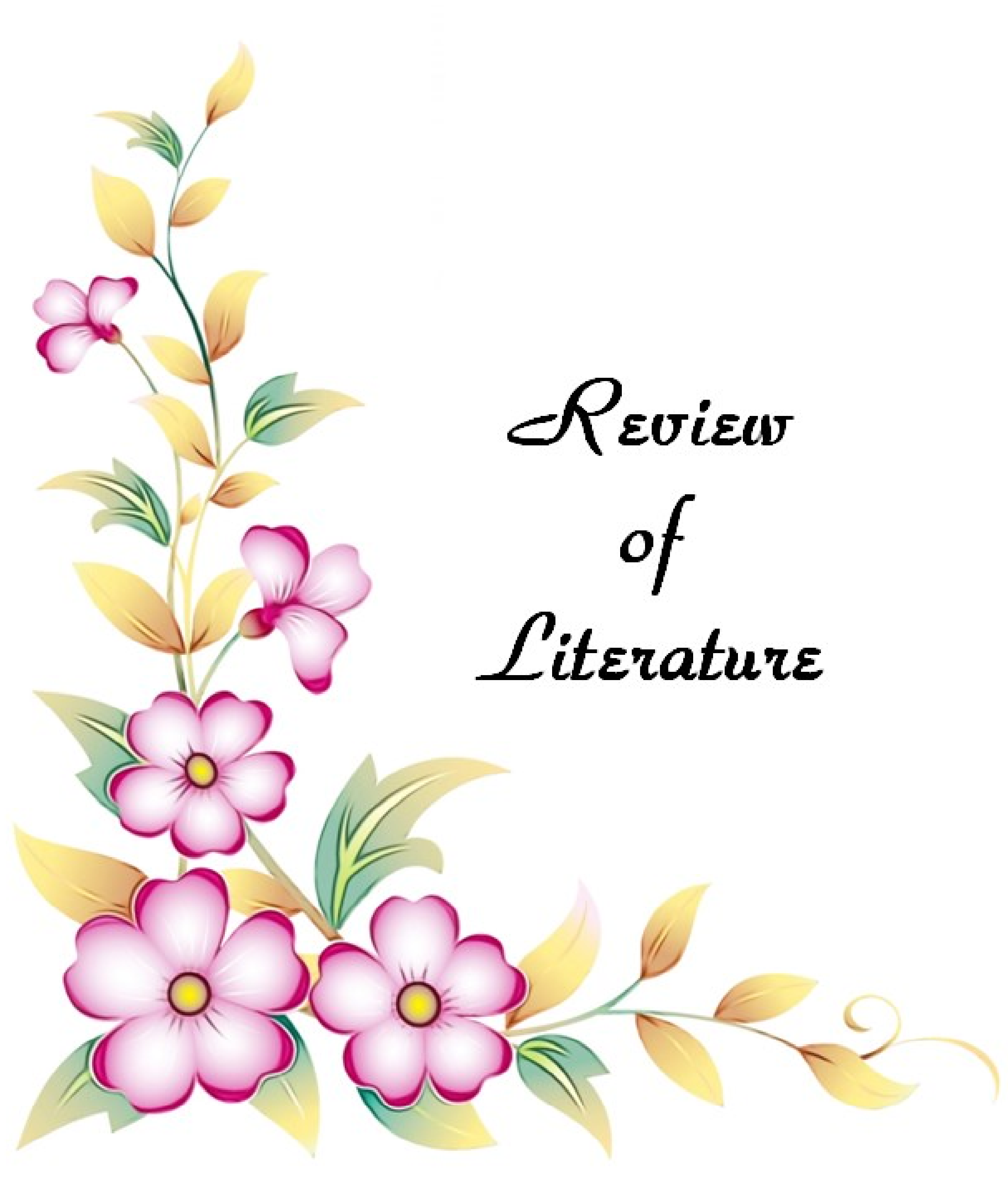
OBJECTIVES

1. **Isolation and characterization of VRE isolated from faecal samples of pig, pork, pig handlers and their environment.**

2. **The relationship between various epidemiological factors and the occurrence of VRE isolated from pigs, pork, pig handlers and their environment.**

✍✍✍

Review of Literature



2 REVIEW OF LITERATURE

The history of enterococci dates back to a century when Thiercelin (1899) used the term "enterocoque" in a French publication to describe bacteria appearing in pairs and also as short chains in human faeces. The *Streptococcus faecalis* name was first coined by Andrewes and Horder (1906) for an isolate found from blood of an endocarditis patient and considering that it was "so characteristic that of human intestinal origin" (Biswas, 2015). *Streptococcus faecium*, a second microbe of this group reported by Orla-Jensen (1919), varied from *S. faecalis* in its fermentation characteristics. Sherman and Wing (1935) hypothesized a third species, *S. durans*, which is identical to *S. faecium* but has less fermentation activity. Sherman pointed out in 1937 that the name *Enterococcus* was used to indicate a variety of things, from a wide definition of any faeces *Streptococcus* to a more narrow zone of organisms that looked to be similar to *S. faecalis*. He established a categorization system for streptococci that divided them into 4 groups: pyogenic, viridians, lactic, and *Enterococcus*. Sherman's classification approach corresponded to Lancefield's serological scheme from the early 1930s (Lancefield, 1933), in which enterococci reacted to group D antisera.

Kalina (1970) proposed a separate genus "*Enterococcus*" based on morphological traits and cellular arrangement for the *Enterococcus* spp. Later Schleifer and Kilpper-Balz (1984) provided genetic evidence using DNA-DNA and DNA-rRNA hybridization to prove that *S. faecalis* and *S. faecium* were sufficiently different from other associates of the genus Streptococci including *S. bovis* and suggested to merit a separate genus. Collins (1998) used DNA homology studies to show that the strains *S. avium*, *S. casseliflavus*, *S. durans*, *S.*

faecalis subspecies *malodorarus* and *S. gallinarum* were closely related to the genus *Enterococcus* and they proposed the new names *E. casseliflavus*, *E. malodoratus*, *E. durans*, *E. avium,* and *E. gallinarum* for those species. As of August 2004, there are 28 species of enterococci proposed with appropriate genetic evidences (Carvalho *et al*., 2004a).

Enterococci are the most commonly reported pathogens in nosocomial surgical-site infections and it is among common pathogens ranking third in nosocomial primary bloodstream infections (BSIs) after coagulase-negative Staphylococci *and Staphylococcus aureus* (Richards *et al*., 2000). *E. faecalis* and *E. faecium* have been identified as the principal enterococcal species responsible for the bulk of human infections. More than 90 percent of clinical isolates come from these two species. Other species of enterococci that includes *E. gallinarum*, *E. durans*, *E. avium*, *E. casseliflavus* and *E. raffinosis* are far less common, accounting for lesser than 5 per cent of clinical isolates. The most prevalent isolates in the human GI system are *E. faecalis* and *E. faecium* (Fisher and Phillips, 2009).

2.1 Habitat and environmental significance of enterococci

Enterococci are found in gut microbiota of reptiles, fish, birds, mammals, plants and insects. Human and animal wastes are disposed to environment through sewage or non-sewage systems. So, presence of *Enterococcus* spp. in food and water indicates faecal contamination. In water, *E. faecium* and *E. feacalis* are used as faecal contamination indicators but other species can also be recovered in less rate. They are readily recovered from foods such as milk and meat products (Blaimont *et al*., 1995). As a result, enterococci have been linked to food deterioration in processed cooked beef. *E. avium, E. durans, E. faecium, E.gallinarum, E. casseliflavus, E. faecalis* and *E. hirae* are commonly isolated from products of cheese and raw meat products (Carvalho *et al*., 2004b). In humans stool enterococci are up to 10^8 Colony Forming Units per gram are the concentrations of enterococci which are representative in stool. Enterococci can colonize oral cavity and also vaginal tract but are only collected from these sites in around 20% of cases (Murray, 1990; Kuhn *et al*., 2003; Mondino *et al*., 2003).

2.2 Prevalence of *Enterococcus* spp

Enterococci have been detected from many domestic animals and wild animals and in domestic and wild birds and they serve as source of infection to humans. Few early reports

have suggested the food poisoning potential of these organisms (Cantoni and Bersani, 1988). Though, there is no general agreement on whether enterococci pose threat as food poisoning organisms, there are reports of animals harbouring antibiotic resistant strains of enterococci suggesting the possibility of spread of these organisms through food chain (Van Den Bogaard *et al*., 1997). Pavia *et al*. (2000) demonstrated widespread dissemination of glycopeptide-resistant enterococci strains in meat in Italy.

2.2.1 Prevalence of enterococci in meat of animal origin

Thal *et al*. (1995) reported that out of 18 enterococci isolates obtained from 29 frozen chicken samples of South Eastern Michigan supermarkets, 11 were *E. faecalis* isolates, *E. faecium* (3), *E. gallinarum* (3) and *E. casseliflavus* (1). Chandra and Garg (2006) analysed total of 37 samples of meat which included chevon (11), Pork (10), chicken (10) and carabeef (6) for presence of enterococci. The positive samples usually carried more than one species of genus *Enterococcus*. A total of 35 enterococci isolates were found, with *E. faecalis* being the most common, followed by *E. gallinarum* and *E. raffinosus*. Other species included *E. faecium, E. pseudoavium, E. hirae, E. cecoruum E. durans, E. mundtii, E. solitarius, E. dispar a*nd *E. avium*.

2.2.2 Prevalence of enterococci in pork

Kim and Koo, (2020) analysed the occurrence of *E. faecium* in retail pork meat products. Pavia *et al*. (2000) reported isolation of 8 (33.3%) enterococci isolates from 24 pork samples taken from retail markets of Catanzaro, Italy. Klein *et al*. (1998) isolated 101 enterococci isolates from minced pork which included *E. faecalis* (85), *E. faecium* (6), *E. casseliflavus* (6), *E. gallinarum* (2), *E. durans* (1) and *E. avium* (1).

2.3 Transmission

The first *Enterococcus* associated foodborne illness was reported in 1926 when two outbreaks of gastroenteritis from cheese were reported (Stiles, 1989). Enterococci were implicated by their occurrence in vast numbers in the incriminated foods and the non-occurrence of other pathogens such as *S. aureus* or *Salmonella* spp (Bryan, 1979). It is felt that food

intoxication can be caused by enterococci by the production of biogenic amines, but both of these observations are yet to be confirmed (Giraffa, 2002).

Water contaminated with enterococci having virulence properties and antibiotic resistance can be a possible source of risk for the consumers (Peter *et al.*, 2012). The sources of enterococci include sewage, plant debris, agricultural runoff, urban runoff, polluted ground water, stormwater, by animal faeces, soils, sediments, and sands. Sewage in developed countries is normally well-treated before being discharged by outfall that is positioned far away from recreational waters. Direct inputs of untreated sewage, however, can impact recreational waters during storm events in regions that have combined sewer overflows and in regions with leaking sewer lines (Sercu *et al.*, 2009).

2.4 Public health significance of Enterococci

MacCallum and Hastings (1899), the people who had identified organism from a patient suffering with acute endocarditis and named it *Micrococcus zymogenes*. They were first to notice enterococci's pathogenic potential. Now, about a 100 years later, enterococci have evolved as significant organisms able to cause a diverse variety of community-acquired and hospital-acquired infections in human beings. In the previous two decades, *Enterococcus* species have particularly acquired a prominent position as emerging pathogens. The NNIS Report declared enterococci to be ranking 2nd to *Escherichia coli* (NNIS Report, 2004). The 3rd most significant cause of nosocomial infection is enterococci, which account for 12.8 percent of all isolates (NNIS Report, 1997). Enterococci are bacteria that cause many ailments. UTI are the most common of these, with the majority of cases being nosocomial (Moellering, 1992). Enterococci are implicated in up to 16% of nosocomial UTI (Schaberg *et al.*, 1991). Wounds, usually intra-abdominal or pelvic are the next to UTI (Moellering, 1992). They are also cause occasional neonatal, CNS and respiratory tract infections (Murray, 1990).

In India, Khanal *et al.* (1998) recorded involvement of HLGR enterococci in IE. Enterococcal infections occur both by endogenous and exogenous route (Moellering, 1992; Morrison *et al.*, 1997) but there are increased recent reports of exogenous spread of MDR enterococci in hospital environment (Moellering, 1992).

Enterococci were regarded as a harmless commensal bacterium and were even believed to have positive effects on a number of gastrointestinal and systemic conditions. However, when the commensal relationship with the host is disrupted, enterococci can cause invasive infections (Lengfelder *et al.*, 2019). Enterococci are now firmly established as one of the major nosocomial pathogens and are increasingly becoming more resistant to antimicrobial agents. Presently, almost all nosocomial enterococcal infections are caused by, either, *E. faecalis*, or *E. faecium* (Dubin and Pamer, 2017). Of these, *E. faecalis* is the most pathogenic species, but *E. faecium* is of increasing importance as, in general, it frequently is more resistant to antimicrobials (Nilson, 2012). Commonly, these organisms are involved in hospital-acquired infections such as catheter-associated urinary tract infections, endocarditis, bacteremia, neonatal sepsis, surgical and burn wound infections, and more rarely meningitis (Dubin and Pamer, 2017). Enterococci are typically harmless in healthy individuals. They become opportunistic pathogens mainly by causing infections in patients who are in Intensive Care Units, who suffer from a severe underlying disease, or who are immunocompromised. Therefore, the severity of illness and immune suppression can be directly associated with prolonged hospital and/or indiscriminate antibiotics use, and these are major risk factors for nosocomial acquisition of drug-resistant enterococci (El-Kersh *et al.*, 2016). Enterococci are the 2nd most common isolates from the skin and soft-tissue infections. Nosocomial infections of wounds caused by surgical, blood stream, the urinary system and other sites are caused by these bacteria (Gilmore *et al.*, 2013; Arias and Murray, 2012).

2.5 Isolation of *Enterococcus* spp.

Because of their importance in food, feed, environmental, and clinical samples, as well as current research activities, the identification and recording of enterococci became a main concern. For various reasons, many media and protocols have been devised, but even single approach is not available that fits all needs uniformly. Depending on the nature of the accompanying microflora and its level, certain substrates and modifications have to be used, taking into account various advantages and drawbacks (Domig *et al.*, 2003).

Garg and Mital (1991) used several media for enterococci isolation from foods and concluded that most of the media showed disadvantages in selectivity and recovery. Kenner

Faecal agar (KF agar) is a good compromise for enumerating enterococci in non-dairy meals, while citrate azide agar is advised for dairy products. The media used to examine enterococci is normally incubated at 35–37°C. When studying enterococci in dairy products, however, a higher incubation temperature (45°C) is required to prevent the background microflora from growing (Deibel and Hartman, 1984). Barton and Doern (1995) compared two selective media (Bile Esculin agar) for the discovery of VRE from GI tract and concluded that it is effective. A modified *Campylobacter* Blood Agar can be used to isolate VRE from stool specimens (Edberg *et al*., 1994). Shigei *et al*. (2002) used a commercially available *Campylobacter* medium supplemented with Vancomycin for screening VRE in clinical samples. The main drawback of this medium is that it supports growth of gram-negative bacilli because of which it is difficult to isolate enterococci. VRE cannot be distinguished on this medium due to lack of characteristic colony morphology of enterococci (Jenkins *et al*., 2011; Devhare *et al*., 2021).

2.5.1 Bile esculin azide (BEA) agar

Enterococci are differentiated easily on BEA agar owing to esculin hydrolysis, which changes the colour of the medium from pale yellow to black. The use of BEA agar with vancomycin combines differential and selective properties to rapidly isolate VRE from heavily contaminated specimens. The process involves significantly less time and resources when compared with conventional method (Jensen, 1996).

Bile esculin hydrolysis is one of the important identification test for *Enterococcus* species. In BEA agar, black colour produced is due to esculin hydrolysis which releases esculetin that combines with iron in the medium to form a black coloured phenolic iron complex (Koneman *et al*., 1997). Presence of azide in the medium inhibits growth of gram-negative bacteria which are predominant bacterial flora of gut. This is the major advantage of using this medium. Other gram-positive cocci like *Staphylococcus* species and diptheroides can grow on this medium but they can be easily distinguished by colony morphology, catalase test and Gram stain (Jenkins *et al*., 2011).

2.6 Biofilm formation by enterococci

Biofilm is secreted by microorganisms in which they are encased or enveloped. Biofilm is extracellular glycocalyx which is protective containing matrix, which can adhere irreversibly to a diverse surface (abiotic and biotic). Antibiotic resistance is higher in biofilm-forming enterococci than in planktonic enterococci. *Enterococcus* has the capacity to bind to various medical devices like ureteral stents, intravascular catheters, biliary stents, ocular lens materials like silicone and acrylic (Stephenson and Hoch, 2002). Biofilm formation genes in the enterococci are *agg, efaA, ace, bop, epbA, epbB, epbC, pil, srt, fsrA, fsrB, fsrC, gelE, sprE*, *cpd, cob and ccf* (Hashem *et al.,* 2017).

2.7 Antimicrobial resistance

The antibiotics usage has started 50 years ago with the leftovers obtained from fermented chlortetracycline which facilitated growth in animals (Guardabassi *et al*., 2004). Antibiotics are used in livestock for three reasons: prevention or prophylaxis of disease encountered by bacterial infection, treatment against serious infections and growth promotion (Schwarz and Chaslus-Dancla, 2001). The usage of antibiotics, persistence of antibiotic residues, and presence of resistant bacteria in the human–animal–environment niches are associated with the One Health triad due to the interdependence of these pillars in the food chain and environment (Aslam *et al*., 2021). The issue of emerging resistant microorganisms associated with livestock is closely linked to improper use of antimicrobial agents in veterinary care, as well as to international trade of food of animal origin, which can contribute to the spread of resistant strains (De Koster *et al*., 2021). Drug-resistant bacteria can circulate among human and animal populations via food, water, and the environment. This transmission is influenced by trade, travel, human migration, and transhumance (Argudin *et al*., 2017; Bonvegna *et al*., 2021).

Antibiotic-resistant bacteria can develop and move between food-producing animals and humans by direct exposure or through the food chain and the environment, irrespective of geographical or ecological borders (FAO,2016). The global rise in meat and meat products demands has led to a shift in farming practices, with a larger proportion of animals projected to

be raised in cost-effective intensive farming systems (Doyle *et al.*, 2006). In such farming systems, the high animal population densities and sub-optimal vaccination, sub-optimal biosecurity, and animal husbandry practices result in the over-reliance on antibiotics for the prophylactic and metaphylactic management of infections resulting in the emergence and spread of antibiotic-resistant bacterial species (Landers *et al.*, 2012; You and Silbergeld, 2014).

Enterococci are less virulent organisms but despite this, enterococcal infections pose serious challenge to the clinicians due to intrinsic and acquired resistance to antibiotics. Enterococci show intrinsic resistance to cephalosporins and less sensitivity to carbapenems, penicillins and other β-lactam agents. These are also insusceptible to aminoglycosides, relatively resistant to lincosamides and resistant *in vivo* to trimethoprim and sulphonamides (Murray, 1990; Morrison *et al.*, 1997). Enterococci are recognised to have acquired genetic determinants, which shows resistance to all classes of antibiotics that includes chloramphenicol, tetracyclines, macrolides, streptogramins, lincosamides, β-lactams, aminoglycosides and most recently to glycopeptides (Panesso *et al.*, 2002).

2.7.1 Multi-drug resistance (MDR) in *Enterococcus* spp.

The increase in resistance to multiple antibiotics in pathogenic bacteria has placed the world at an alarming disadvantage in terms of chemotherapy. Though various definitions exist, in strictest sense, MDR is termed as acquired resistance towards more than three classes of antibiotics and organisms which exhibit MDR are termed as MDR organisms (Kruperman, 1983).

Schlegelova *et al.* (2002) reported that enterococci isolates from bulk milk samples were mainly resistant to tetracycline, streptomycin and erythromycin. Their observation included 88% of *E. faecalis* and 55% *E. faecium* isolates resistant to one or more commonly used drugs.

Peters *et al.* (2003) investigated the resistance patterns to 13 antimicrobial active compounds to 118 *E. faecium* and *E. faecalis* and they found Ampicillin and amoxicillin/ clavulanic acid resistance in strains studied. Only one *E. faecium* strain was shown resistant to penicillin and other strains showed sensitivity to teicoplanin and vancomycin. Most of isolates were resistant to tetracycline, quinupristin/dalfopristin and erythromycin.

Messi *et al*. (2006) investigated antibiogram profile of 178 enterococci isolates of meat (59) and environmental origin (119) which revealed a little incidence of β lactamic resistant strains, whereas streptomycin resistance observed was high (85.7 per cent and 92.8 per cent), kanamycin (79.7 per cent and 96 per cent) and gentamicin (85.1 percent and 91.7 percent) and an intermediate level resistant pattern appeared for erythromycin (35.1% and 10.5%, respectively).

Kimiran-Erdem *et al*. (2007) examined 100 *Enterococcus* strains against 10 antibiotics. None of the strains were resistant to vancomycin and 98% isolates were resistant to nalidixic acid, 88% to streptomycin, 50% to kanamycin, 25% to amikacin, 7% to erythromycin, 6% to ampicillin, 3% to chloramphenicol and 2% each to gentamicin and penicillin G.

Singh (2009) investigated 267 enterococci isolates from 19 clinical and 23 nonclinical samples of equine for antibiogram profile against antimicrobial agents by disc method. Out of 267 isolates, 80.2 percent of enterococci were vancomycin resistant and 99.6 percent of isolates were said to be MDR.

Krocko *et al*. (2011) reported that, out of 75 enterococci isolated from pork, raw beef and poultry, 56, 15, 15, 27 and 25 per cent to were shown resistant to tetracycline, vancomycin, erythromycin, ampicillin and gentamicin respectively. They also discovered that intermediate resistance to antibiotics was more widespread in swine and poultry isolates showing ampicillin (70 percent and 40 percent, respectively), gentamicin (66 percent and 40 percent), tetracycline (54 percent pork only), and erythromycin (64 percent only pork).

Novais *et al*. (2013) reported that, out of 171 *E. faecium* and *78 E. faecalis* isolated from pig farm environment *E. faecium* was more frequently resistant to ampicillin, ciprofloxacin or nitrofurantoin and *E. faecalis* to tetracyclines, chloramphenicol or aminoglycosides.

Enayati *et al*. (2015) observed the resistant pattern of *Enterococcus* isolates recovered from surface water to be 41.5, 27.1, 12.7, 6.8 and 1.7% resistant to tetracycline, erythromycin, ampicillin, ciprofloxacin and chloramphenicol respectively. *E. faecium* in total isolates were sensitive to vancomycin, linezolid, gentamicin, teicoplanin, and quinuspristin- dalfopristin.

Lata *et al*. (2016) reported that out of 60 enterococci isolates recovered from Gomati River, 29 percent isolates showed resistance to erythromycin, macrolide and rifampicin in association with tetracycline.

Nowakiewicz *et al*. (2017) reported that all selected strains from 90 pig samples were susceptible to ampicillin and vancomycin and resistant to tetracycline, chloramphenicol, linkomycin, high-level kanamycin and streptomycin, as well as macrolides (erythromycin and tylosin) and glycopeptides (quinpristin-dalphopristin).

Tan *et al*. (2018) reported that out of 289 enterococci isolated among *E. faecalis* strains, 98% and 96% of them were susceptible to ampicillin and penicillin, respectively. However, among *E. faecium* were more resistant to ampicillin (49%) and penicillin (59%) compared to *E. faecalis*. All the strains were susceptible to vancomycin and only two percent of strains from each species were resistant to teicoplanin.

Igbinosa and Raje (2019) reported that out of 64 enterococci isolates recovered from abattoirs, 64 (100%) were resistant to ertapenem, cefuroxime and cefotaxime. In addition, 54 (84.4%) were resistant to meropenem, 51 (79.7%) were resistant to penicillin, 34 (53.1) were resistant to tetracycline, 27 (42.2%) were resistant to piperacillin, 24 (37.5%) were resistant to clindamycin, and 23 (35.9%) were resistant to vancomycin.

Nowakiewicz *et al*. (2020) reported that the results showed that almost half of the tested wild animals (48%) were carriers of at least one multidrug resistant *E. faecalis* strain. A significant percentage of strains were resistant to high-level aminoglycosides (from 20% to even 57.5%).

Abreu *et al*. (2022) reported that out of 224 samples from pigs no vancomycin resistant enterococci were observed.

Azizi *et al*. (2022) reported that out of 108 enterococci isolates, the rate of resistance to ampicillin, penicillin, gentamicin, erythromycin, and ciprofloxacin norfloxacin, among *E. faecium* isolates, was 91.66%, 83.33%, 75%, 83.33%, 66.66%, and 58.33%, respectively. Meanwhile, *E. faecalis* isolates exhibited 5.81%, 5.81%, 33.72%, 54.65%, 40.69%, and

34.88% resistance to ampicillin, penicillin gentamicin, erythromycin, ciprofloxacin, and norfloxacin, respectively.

Gonzalez-Fandos *et al.* (2022) reported that a higher number of vancomycin-resistant *E. faecium* were recovered compared to *E. faecalis*.

2.7.2 Vancomycin resistance in *Enterococcus* spp.

Vancomycin is considered one of the last lines of treatment against multidrug-resistant bacteria, including ampicillin resistant enterococci (Wijesekara *et al.*, 2017). *vanA* and *vanB* are the most common genotypes of vancomycin resistant enterococci (VRE) (especially among *E. faecalis* and *E. faecium*) in humans and animals (Miller *et al.*, 2014). Evidence suggests a rapid increase in VRE infections in hospitalized patients (Melese *et al.*, 2020). The emergence of VRE in food-producing animals has been thought to be a consequence of the use of avoparcin (a vancomycin analog) in their feed as a growth promoter (Ahmed and Baptiste, 2018). This can result in VRE contamination in food of animal origin and may create a potential risk for human health.

Antibiotics are frequently used as growth boosters in animal farms, which exacerbates the problem. Resistance to vancomycin is either intrinsic or acquired through the possession of nine types of vancomycin resistance genes (*vanA*, *vanB*, *vanC*, *vanD*, *vanE vanG,van L,vanM and vanL)* (Miller *et al.*, 2014). The glycopeptide resistant enterococci were first reported in UK in 1986 (Leclercq *et al.*, 1988), which is variable phenotypically and genotypically.

In 1988, the first case of vancomycin-resistant enterococci (VRE) was reported in Europe (Courvalin, 2006). In India, the first VRE case was reported in New Delhi in 1999, and the prevalence reports of VRE in India vary from 1 to 8.7% (Purohit *et al.*, 2017). The World Health Organization recognized VRE as one of the most significant resistant bacteria in their "Global Priority list of antibiotic-resistant bacteria" in 2017 (Tacconelli, 2017). The Centers for Disease Control and Prevention reported that VRE has caused 54,500 infections among hospitalized patients and 5,400 estimated deaths in the United States in 2017 (CDC, 2017). The European Antimicrobial Resistance Surveillance Network reported the increasing trend

of the mean proportion of vancomycin-resistant *Enterococcus faecium* in invasive isolates from 10.4% in 2014 to 17.3% in 2018 in countries of the European Union and European Economic Area (Ayobami *et al*., 2020). Though the rate of isolation of VRE is currently not very high in India when compared to other countries (USA and Europe), still it is definitely on an increasing side (Vidyalakshmi *et al*., 2012). It was found that the mortality and morbidity are more with VRE bacteraemia (Piezzi *et al*., 2020).

There is so far no composite data available about the trend in India, but as per sporadic reports, the prevalence of vancomycin-resistant enterococci (VRE) in India was about one per cent in 2003, which got inflated up to 8.7 per cent in 2013 (Mamthora *et al*., 2019). Similarly increase in prevalence has been reported from Maharashtra, UP, and Sikkim (Shinde *et al*., 2012; Tripathi *et al*., 2016; Yangzom and Singh, 2019). Studies done in India have shown the prevalence of *vanA*, *vanB* and *vanC* among the enterococci isolated from clinical samples (Phukan *et al.,* 2016).

The emergence of Vancomycin-susceptible *E. faecium* into Vancomycin-resistant *E. faecium* occurs in human gut in antibiotic treatment (Howden *et al.,* 2013). In immunocompromised patients vancomycin resistant *E. faecium* (VREF) has evolved as significant worldwide reason for nosocomial infections (Top *et al*., 2008). vancomycin susceptible enterococci reported to change to vancomycin resistant enterococci during the therapy resulting in resistance. (Thaker *et al*., 2015).

2.7.2.1 *vanA* glycopeptide resistance

In several *Enterococcus* spp *vanA* phenotype glycopeptide resistance acquired by inducible high range of vancomycin resistance (MICs 64mg/L->1000mg/L) and also teicoplanin (MICs 16mg/L- 512mg/L) had been reported (Arthur and Courvalin, 1993; Cercenado *et al*., 1995; Rosato *et al*., 1995) and in certain VR *Staphylococcus aureus* (VRSA) isolates (CDC, 2002). *vanA* is the most completely understood type of glycopeptide resistance (Arthur *et al*., 1996a).

2.7.2.2 *vanB* glycopeptide resistance

vanB phenotype glycopeptide resistance is combined with acquired inducible low to high level resistance (MIC 4mg/L-1000mg/L) to various concentrations of vancomycin but typically not to teicoplanin (MIC 0.25mg/L- 2mg/L) (Zirakzadeh and Patel, 2005). A few isolates with resistance also to teicoplanin have been described (Murray, 2000). *vanB* gene cluster has received less attention than *vanA*. *vanB* is mostly present in *E. faecium* and *E. faecalis* (Arthur *et al*., 1996b).

2.7.2.3 *vanC* glycopeptide resistance

Unlike *vanA* and *vanB* resistance, which can be acquired and transferred, the *vanC* type of resistance was found in *E. gallinarum*, *E. casseliflavus*, and *E. flavescens* as an innate constitutive feature (Cercenado *et al*., 1996). This type of resistance is characterized as resistant to vancomycin (MIC 8-32 μg/ml) but sensitivity to teicoplanin (8 μg/ml).

2.8 Molecular detection of VRE

Since vancomycin resistance genes are transferable among different enterococci species (Woodford *et al.,* 1995), the lack of prompt detection of enterococci may cause interruption in attempting to prevent VRE colonization and infection. Conventional identification methods for enterococci require at least two to three days. The development of rapid, sensitive PCR-based assays has improved the accuracy and speed of the diagnosis of enterococcal infections. PCR provides a means for culture- independent identification of enterococci in many clinical specimens and can produce results of identification in few hours. Many PCR-based techniques can detect VRE (Dutka-Malen *et al*., 1995; Clark *et al*., 1998; Ke *et al*., 1999). Biswas *et al*. (2016) reported that out of 500 *Enterococcus* isolates studied, 60 VRE/VIE isolates were detected by MIC and were also confirmed to carry (*vanA)*(vanB*)* genes by PCR.

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Materials and Methods

3 MATERIALS AND METHODS

The objectives of this study were to isolate and identify *E. faecium* and *E. faecalis* from pigs, pork and their environment (Water, Soil, Feed, Sewage); their phenotypic and genotypic as well as characterization for Vancomycin resistance along with determining the association of different epidemiological factors. The samples were collected from 5 organized farms located in Bareilly district (U.P.) and 3 organized farms located in Krishna district (A.P.). The study was conducted in the Division of Epidemiology, Indian Veterinary Research Institute (IVRI), Izatnagar, India.

3.1. General experimental materials

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

The details of media and chemicals used in the study are given in the appendix. The media and antibiotic discs used in study were procured from Hi-Media (Mumbai, India). The PCR Master Mix was procured from Thermo Scientific, USA. The PCR primers were custom synthesized from Eurofin Pvt. Ltd., Bangaluru, India. All media and reagents for molecular biology work were prepared in triple distilled water and for isolation and culture of bacteria in single glass distilled water.

3.1.2 Plasticware and glassware

Plasticware (beakers, measuring cylinders, Eppendorf tubes and holders, 15 ml and 50 ml tube holders, floaters, disposable sterile Petri plates) used in this study were from Tarsons (India). All glass items (beakers, measuring cylinders, bottles of different capacities) were from Schott Duran. Test tubes of various sizes were procured from Borosil, India. Glassware's were thoroughly washed and sterilized wherever necessary as per the standard procedures.

3.1.3 Equipments

Equipments used in the study were autoclave (Scientific Equipments Works, New Delhi, India), bio-saftey cabinet (Kartos International, Noida, India), cooling centrifuge (REMI Instruments Ltd., Mumbai, India), deep freeze -20°C (Vestfrost), electronic balance (Mettler Toledo, Switzerland), gel documentation system (AlphaInnotech Co., USA), incubator (Khera Instruments Pvt. Ltd., New Delhi, India), variable volume micro pipettes (Finnpipette), water bath (Narang Scientific Works Pvt. Ltd., New Delhi, India), PCR thermal cycler (MJ Research PTC-200, Peltier Thermal Cycler, USA), UV illuminator (Crystal BioGlow, China), hot air oven (Scientific Equipments Works, New Delhi, India).

3.2 METHODS

3.2.1 Collection of faecal samples

Pig faecal samples were collected from pig farms of Bareilly district (U.P) and Krishna district (A.P). Faecal samples from the rectum of pigs were collected using (Hi-Media) sterile transport swabs and transferred to buffered peptone water for pre-enrichment. The pork samples were collected from Bareilly district (U.P) in sterile beakers and transferred to buffered peptone water (BPW) for pre-enrichment. The details of samples collected are given in table 3.1and table 3.2.

Table 3.1: Details of sample collected from organized pig farms in Krishna district (AP) and Bareilly district (UP)

S. No	Place	No of samples	Farm Wise collection	Type of Samples
1	Krishna district (Andhra Pradesh)	75	Farm-1 (n=30)	F (20), E (8), H (2)
			Farm-2 (n=25)	F (15), E (8), H (2)
			Farm-3 (n=20)	F (15), E (4), H (1)
2	Bareilly district (Uttar Pradesh)	125	Farm-1 (n=40)	F (25), E (10), H (5)
			Farm-2 (n=30)	F (15), E (10), H (5)
			Farm-3 (n=20)	F (13), E (3), H (4)
			Farm-4 (n=20)	F (12), E (5), H (3)
	Total	200	Farm-5 (n=15)	F (10), E (2), H (3)

F- Faecal swabs, E-Environmental Samples (Water, Soil, Feed, Sewage), H-Human hand swabs

Table 3.2: Details of pork samples from retail meat shops in Bareilly district of UP

S. No	Place	No: of samples (n=50)
1	Delapeer	15
2	Naikpur	15
3	Satellite road	10
4	Subhash nagar	10
5.	Total	50

3.3 Processing of the samples

The meat samples (10 gram) were processed by pre-enrichment in the buffered peptone water (90 ml) in the ratio of 1:10 overnight in a sterile glass conical flask followed by their streaking on the selective Bile esculin azide agar (BEA) plates and incubated for 24h at 37°C. The butcher's and animal handlers' hands' swabs were directly streaked over selective BEA plates followed by incubation for 24 h at 37°C. The faecal and environmental samples (water, Soil, feed and sewage) were enriched in BPW in the dilution of 1:10 overnight followed by streaking over selective BEA plates followed by incubation for 24 h at 37°C.

3.3.1 Examination of Colony Morphology of Bacteria

Morphology of bacterial colonies was observed after 24 h of growth at 37°C. The black coloured colonies were picked up and stored till further identification. From each sample, a single typical isolated colony was picked up, given that the morphology of the colonies was identical.

3.3.2 Purification of the *Enterococcus* isolates

For the purification, the primary isolated colonies grown over the selective BEA plates re-streaked over the fresh BEA and incubated for 24 h at 37°C.

3.4 Biochemical characterization of Enterococci isolates

Enterococci isolates were preliminary identified by biochemical tests viz., Catalase, oxidase, Methyl-red, Voges Proskaeur, Motility Indole Lysine (MIL), Nitratase test and production of H_2 S on TSI.

3.4.1 Gram staining

A smear of bacterial culture was made on a clean and grease-free glass slide using a sterile loop. The Gram staining was performed as per standard protocol (Bartholomew and Mittwer, 1952). The slide was air-dried and was examined under the microscope at 40x and 100x magnifications with immersion oil. The putative *Enterococcus* cultures were examined for microscopic cell shape of gram-positive cocci.

3.4.2 Catalase test

On a glass slide, a drop of hydrogen peroxide (3% H_2O_2) was mixed with a loop full of bacterial culture. Production of the bubbles (immediately) on the glass slide was indicative of catalase-positive reaction.

3.4.3 Oxidase test

A loop-full of bacterial growth was rubbed onto commercially available oxidase discs (Hi-Media) and appearance of deep purple colour within 20-30 seconds was indicative of oxidase production.

3.4.3 Methyl Red (MR) and Voges Proskauer (VP) test

A loopful of culture was inoculated in to a test tube containing 5ml MR-VP broth (Hi-media Laboratories Ltd., Mumbai) and incubated at 37°C for 48h. First MR test was conducted through adding 1-2 drops of MR reagent, development of red colour indicates positive test. For VP test, in the same tube, 0.3 ml of 5% alpha napthol in absolute ethyl alcohol and then 0.1 ml of 40% (w/v) KOH was added. A positive reaction was noticed with development of bright red colour within 15 min, while no colour change indicated a negative reaction.

3.4.4 Motility indole lysine medium

MIL medium (Hi-Media Laboratories Ltd., Mumbai) was used for detection of motility, indole production, lysine decarboxylation and H_2S production. A small fraction of one colony of enterococci was taken on inoculation needle and stabbed into the tube containing MIL media; oxalic acid strip was kept in tube and incubated at 37°C for 24h. Non-motile organism

grows along stabbed line while motile bacteria form diffused growth. H_2S production was indicated by blackening of the medium. Lysine decarboxylase is indicated by a purple colour throughout the medium. This colour may vary in intensity and may be bleached out to a pale light colour due to reduction of the indicator. Lysine-negative cultures produce a yellow medium that may be purple or red on the top. Reddish colour of oxalic acid strips on the top of tubes shows positive for indole test.

3.4.5 Hydrogen Sulphide production on Triple Sugar Iron (TSI) Agar

The TSI agar (Hi-Media Laboratories Ltd., Mumbai) slants were inoculated heavily by stabbing the butt and streaking the slope of slant. The tubes were incubated at 37°C for 24h and observed. Black discolouration of butt and slant indicated an H_2S production while, yellow colour of butt and slant indicated acid production.

3.4.6 Nitrate reduction

A loopful of culture was inoculated in nitrate broth and then incubated at 37°C for 24h. Reduction of nitrate to nitrite was detected by adding 0.1 ml of sulphanilic acid and 0.1 ml of an α-naphthylamine reagent to broth culture, the presence of nitrite was indicated by the appearance of red colour. If a red colour does not develop, a pinch of zinc dust was added and observed for the appearance of red colour in case of negative test otherwise the test was considered positive.

3.5 Phenotypic characterization for virulence

3.5.1 Biofilm quantification assay

The biofilm quantification assay was done using a previously described (Stepanvic *et al*., 2000) technique with a little modification, 50 g/L sucrose in BHI broth to increase the biofilm formation. The *E. faecium* and *E. faecalis* isolates were inoculated in BHI broth for 24 h at 37ºC. Followed by microtitration, 200 µL of the bacterial suspensions were poured in triplicate on polystyrene plates containing 96 flat-bottom wells, and BHI broth without bacterial inoculum was used as the negative control. The microtiter plates were further incubated at 37ºC for 24 h. The bacterial suspensions were removed and wells were washed three times, with 250 µL of sterile saline solution (0.9% NaCl).

Subsequently, the plate was fixed with 200 µL of ethanol for 15 minutes. The ethanol was removed, the plates were left at room temperature to dry, and then, microtiter plate was stained with 200 µL of 2% crystal violet solution for 5 min. Then, microtiter plates were washed under running tap water and air dried at room temperature. The absorbance reading of crystal violet was taken in an ELISA reader (Bio-Rad, model 550) at a wavelength of 570 nm, and samples were classified according to (Stepanovic *et al.*, 2000). The average of triplicate optical was considered as the final value for each isolate (ODi), and this value was compared to the optical density of the negative control (ODc). The strains were classified into four categories, according to the mean optical densities (OD).

Table 3.3: Criteria for classifying biofilm formation ability of enterococci based on the optical density value

Biofilm ability	Comparison with OD	Classification
Non adherent	OD<ODc	-
Weak	ODc<OD ≤2x ODc	++
Moderate	2x ODc < OD ≤4x ODc	+++
Strong	4x ODc< OD	++++

3.5.2 DNase test

DNase agar (Difco) plates were spot inoculated with test organisms and incubated for 24-48 h. Clear halos around the bacterial growth indicates positive for DNase production.

3.5.3 Hemolysis test

It was assessed by streaking on 5% sheep blood agar plates and incubated at 37°C for 24h. Zone of hemolysis around the colonies indicates positive reaction.

3.6 Molecular confirmation of *E. faecium* and *E. faecalis* by PCR

All *Enterococcus* isolates were subjected to *Enterococcus* species specific PCR assay for further molecular confirmation. The bacterial DNA with absorbance (A) ratio (A260/ A280) of 1.8 to 2.0 was used as template for PCR reaction. The *Enterococcus* isolates that were positive by cultural and biochemical tests, 130 were confirmed genotypically by species specific PCR as *E. faecium* and *E. faecalis.*

Table 3.4: The details of primers and amplicon size for specific detection of *E. faecium* and *E. faecalis* using Duplex PCR

Oligonucleotide primers	Sequence 5' to 3'	Target gene	Amplicon size (bp)	Reference
E. faecium	F-5'-ACTTATGTGACTAACTTAACC-3'	*SodA*	215	Jackson *et al*., 2004
E. faecalis	R-5'-TAATGGTGAATCTTGGTTTGG-3'	*SodA*	360	Jackson *et al*., 2004

3.6.1 Isolation of DNA from Enterococci isolates

Selected isolates of Enterococci were inoculated in 5 ml of LB broth and incubated at 37°C for overnight. A loop full of the broth was streaked on nutrient agar plate followed by incubation at 37°C for overnight. A single colony was picked and inoculated again in fresh 5 ml of LB broth and then incubated at 37°C for 6-8 h. The bacterial suspension was subjected to DNA isolation using snap chill method. The concentration of DNA was measured in Nanodrop (Eppendorf), and the DNA was finally stored at -20°C in small aliquots.

3.6.2 Steps for isolation of genomic DNA from bacterial suspension cultures

1. The culture was prepared by inoculating the isolate in Luria-Bertani broth and incubating at 37°C for 24h.
2. The cultured broth was streaked on Nutrient agar and incubated at 37°C for 24h. Loopful of 24h grown culture on Nutrient agar was suspended in 200µl of nuclease free water in a 1.5ml micro centrifuge tube.
3. After mixing properly, the tubes were heated to 100°C in water bath for 15min and immediately placed on ice (-20°C).
4. After 20 min the bacterial lysate was centrifuged at 13,000rpm for 10min and the supernatant was used as DNA template for PCR assays.

3.6.3 Molecular confirmation of *Enterococcus* strains at the species level

The molecular confirmation of *E. faecium* and *E. faecalis* was done by using species-specific primers (Table 3.4). The amplification of targeted DNA was carried out in 25 µl reaction volumes, containing 1 µl each of the two primers, 5 µl of 2x Master mix (Thermo

scientific) and 3 µl of bacterial DNA, and adjusted to 25 µl by the addition of molecular biology grade nuclease-free water. The PCR tubes with all reaction were transferred to 96 well thermal cyclers. Amplification was done on a Thermocycler (Eppendorf). The thermocycling details are given in Table 3.5. The amplified product was examined in 1.5% agarose gel prestained with ethidium bromide. The product was visualized under UV light, and results were documented.

Table 3.5: The steps and cyclic conditions for PCR of *E. faecium* and *E. faecalis* isolates

Steps	Action	Temperature (°C)	Time	No. of cycles
1.	Initial denaturation	95	8 min	
2.	Denaturation	95	30 sec	30
3.	Annealing	50	1 min	
4.	Extension	72	1 min	
5.	Final extension	72	7 min	

3.6.4 Agarose gel electrophoresis

The confirmation of targeted PCR amplification was done by using Agarose gel electrophoresis. The gel was prepared by using 1% Agarose in 1× Tris-acetate-EDTA (TAE) buffer, pH 8.3 (Thermo Fisher Scientific) and ethidium bromide (1%). About 6µl of PCR product was slowly loaded into the wells. 6 µl of 100 bp DNA ladder was loaded as molecular weight marker (GeneRuler 100 bp plus, Thermo Scientific) in one well. For 1 hour, samples were run at 90 V in 1x Tris acetate-EDTA (TAE buffer) (Thermo Scientific). The amplified PCR product was visualized under a gel documentation system, as a single compact band of the expected size.

3.7 Assay for antimicrobial drug resistance of the *E. faecium* and *E. faecalis* isolates

The bacterial isolates were subjected to *in vitro* antibiotic sensitivity test by disc diffusion method (CLSI, 2020) on Muller-Hinton agar (MHA) plates (Bauer, 1966). The antibiotic discs were obtained from (Hi-Media Laboratories Ltd., Mumbai). Isolates were tested against commonly used antibiotics viz. Vancomycin (30µg), erythromycin (15µg),

gentamicin (10µg), tetracycline (30µg), ampicillin (10µg), amoxicillin (25µg), oxacillin (5µg), Penicillin G (10 Units), teicoplanin (30µg), nitrofurantoin (300µg), ciprofloxacin (5µg), linezolid (30µg), high level streptomycin (300µg), azithromycin (15µg) and rifampin (5µg).

Isolates were grown in Luria-Bertoni broth overnight and lawn cultures were prepared on Mueller Hinton Agar (MHA) plates. The plates were allowed to dry. Antibiotic discs were placed on agar surface at about two cm apart. The plates were incubated at 37°C overnight and diameter of the zones of inhibition was measured. The measurements were compared with zone size interpretative chart furnished by the manufacturer and the zones were graded as sensitive, intermediate and resistant.

Table 3.6: List of antimicrobials used for antimicrobial susceptibility testing against *E. faecium* and *E. faecalis* isolates

S.No.	Antimicrobial agents	Disk content (µg)	Sensitivity (mm)	Intermediate (mm)	Resistance (mm)
I.	**Penicillins**				
1	Ampicillin (AMP)	10	≥17	14-16	≤16
2	Amoxicillin (AMX)	10	≥18	14-17	≤13
3	Pencillin(P)	10	≥15	-	≤14
4	Oxacillin(O)	5	≥17	-	≤14
II.	**Glycopeptides**				
5	Vancomycin (V)	30	≥17	15-16	≤14
III.	**Lipoglycopeptides**				
6	Teicoplanin (TEI)	30	≥14	11-13	≤10
IV.	**Aminoglycosides**				
7	Gentamicin (GEN)	10	≥15	13-14	≤12
8	Streptomycin (HLS)	300	≥10	7-9	≤6
V.	**Tetracyclines**				
9	Tetracycline (TE)	30	≥19	15-18	≤14
VI.	**Fluoroquinolones**				
10	Ciprofloxacin (CIP)	5	≥21	16-20	≤15
VII.	**Nitrofurantoins**				
11	Nitrofurantoin (N)	300	≥17	15-16	≤14
VIII.	**Ansamycins**				
12	Rifampin (RIF)	5	≥20	17-19	≤16
IX.	**Macrolides**				
13	Azithromycin (AZM)	15	≥12	-	≤11
14	Erythromycin(E)	15	≥23	14-22	≤13
X.	**Oxazolidinones**				
15	Linezolid (L)	30	≥23	21-22	≤20

3.7.1 Determination of Minimum inhibitory concentration (MIC) of vancomycin for enterococci isolates

MIC was performed with Microbroth dilution method (CLSI, 2020). Stock solutions of the antibiotics were prepared and stored at -20°C.Working solution of antibiotics were prepared with Muller Hinton (MH) broth.100 µL of MH broth was added to all the wells in 96 well plates.100 µL of antibiotic solution was added to first well and serial dilution of the antibiotics was done till 10th well. Then 2 µL of culture was added till 11th well and the plates were incubated at 37°C for 24h.

Table 3.7: MIC determination

Wells	1	2	3	4	5	6	7	8	9	10	11	12
Antibiotics (µg/mL)	128	64	32	16	8	4	2	1	0.5	0.25	Growth control	Broth control

3.8.1 Isolation of DNA from *E. faecium* and *E. faecalis* isolates

Isolates of *E. faecium* and *E. faecalis* that were showing resistance to vancomycin by Disc-diffusion and MIC (Minimum inhibitory concentration) were inoculated in 5 ml of LB broth and incubated at 37°C for overnight. A loop full of the broth was streaked on nutrient agar plate and incubated at 37°C for overnight. A single colony was picked and inoculated again in fresh 5 ml of LB broth and then incubated at 37°C for 6-8 h. The bacterial suspension was subjected to DNA isolation using snap chill method. The concentration of DNA was measured in Nanodrop (Eppendorf), and the DNA was finally stored at -20°C in small aliquots. The steps for isolation of DNA were mentioned in 3.6.2.

3.8.2 Molecular detection of VRE strains

The molecular detection of VRE was done by using specific primers (Tab 3.8). The amplification of targeted DNA was carried out in 25 µl reaction volumes, containing 1 µl each of the two primers, 5 µl of 2x Master mix (Thermo scientific) and 3 µl of bacterial DNA, and adjusted to 25 µl by the addition of molecular biology grade nuclease-free water. The PCR tubes with all reaction were transferred to 96 well thermal cyclers. Amplification was done on a Thermocycler (Eppendorf). The thermocycling details are given in Table 3.9. The amplified

product was examined in 1.5% agarose gel prestained with ethidium bromide. The product was visualized under UV light, and results were documented.

Table 3.8: Details of primers used for PCR reaction for detection of vancomycin resistant enterococci (VRE)

Sl. No.	Sequence 5' to 3'	Target gene	Amplicon size (bp)	Reference
1	F-5'-GCGCGGTCCACTTGTAGATA-3' R-5'-TGAGCAACCCCCAAACAGTA-3'	*vanA*	314	Nam *et al.*,2013
2	F-5'-AGACATTCCGGTCGAGGAAC-3' R-5'-GCTGTCAATTAGTGCGGGAA-3'	*vanB*	220	Nam *et al.*,2013
3	F-5'- ATCCAAGCTATTGACCCGCT-3' R-5'- TGTGGCAGGATCGTTTTCAT-3'	*vanC1*	402	Nam *et al.*,2013

Table 3.9: The steps and cyclic conditions for PCR of *vanA, vanB* and *vanC1* genes

Steps	Action	Temperature (°C)	Time	No. of cycles
1.	Initial denaturation	94	5 min	
2.	Denaturation	94	1 min	35
3.	Annealing	54	1 min	35
4.	Extension	72	1 min	35
5.	Final extension	72	15 min	

3.8.3 Agarose gel electrophoresis

The confirmation of targeted PCR amplification was done by using Agarose gel electrophoresis. The gel was prepared by using 1% Agarose in 1× Tris-acetate-EDTA (TAE) buffer, pH 8.3 (Thermo Fisher Scientific) and ethidium bromide (1%). About 6μl of PCR product was slowly loaded into the wells. 6 μl of 100 bp DNA ladder was loaded as molecular weight marker (GeneRuler 100 bp plus, Thermo Scientific) in one well. For 1 hour, samples were run at 90 V in 1x Tris acetate-EDTA (TAE buffer) (Thermo Scientific). The amplified PCR product was visualized under a gel documentation system, as a single compact band of the expected size.

3.9 Statistical analysis

Differences among the rates of antibiotic resistance and among isolates recovered from different samples were analysed by chi-square test using SPSS software, 28.0 version (SPSS Inc., USA).

✍✍✍

Results

4 RESULTS

The present study was conducted to determine the occurrence of the vancomycin resistance (VRE) in commensal *Enterococcus* isolated from pig farms, Pork and their environment. The isolates were assayed for their biochemical characters, antibiotic susceptibility pattern, vancomycin resistance by phenotypic and genotypic detection and also to determine the association of various epidemiological factors with *Enterococcus* isolated from organized pig farms located in Bareilly district (Uttar Pradesh) and Krishna district (Andhra Pradesh).In the present study a total of 250 samples comprising of faecal swabs (n=125), environmental samples which included Water, Soil, Feed and Sewage (n=50), human hand swabs (n=25) and pork (n=50) were collected and processes for isolation of *Enterococcus*..

4.1 Isolation, cultural identification and characterization of *Enterococcus* isolates

4.1.1 Isolation of enterococci isolates

For isolation of enterococci, Bile Esculin Azide agar (BEA) is used as a selective plating media. All the samples were pre- enriched in bufferd peptone water for 24h at 37°C followed by plating on Bile Esculin Azide agar (BEA)and incubated for 24h at 37°C for isolation of *Enterococcus* colonies. Enterococci isolates were selected based on specific colony characteristics viz., small transparent colonies with brown-black halos (Fig 4.1). Out of 250 samples analysed, 160 (64%) samples were found to be positive for *Enterococcus* spp. by cultural isolation and on the basis of species-specific polymerase chain reaction (PCR) 130 were found to be *E. faecium* (67) and *E. faecalis* (63). The isolates were subjected to phenotypic characterization of virulence by conducting biofilm formation, DNase tests and hemolysis test. The antibiogram studies of 130 *Enterococcus* isolates had been carried out

using standard disc diffusion assay. The molecular typing studies of the VRE genes had been carried out by Polymerase chain reaction.

4.1.2 Characterization of enterococci isolates

The characterization of isolates was carried out primarily by Gram's staining, oxidase test, and catalase test. All 160 isolates displayed the microscopic appearance of Gram's positive cocci on staining and appeared as blue/violet stained small or medium-sized cocci. (Fig 4.2) when visualized under an oil immersion lens. All isolates were oxidase negative and gave a negative catalase test result (Table 4.1).

Table 4.1: Primary biochemical tests carried out for *Enterococcus* confirmation

S. No.	Test	Positive	Negative
1	Oxidase test	Blue colour	No colour appearance
2	Catalase test	Bubble production	No bubble production

4.1.2.2 Secondary biochemical tests

The secondary biochemical tests included the Methyl Red test, Voges-Proskauer test, MIL, TSI and Nitrate test. The results of secondary biochemical tests are summarized in Table 4.2 and Fig 4.3. After phenotypic testing, the isolates were identified as *Enterococcus.*

Table 4.2: Secondary biochemical tests

S. No.	Test	Positive result	Negative result
1	Methyl-red test	Dark red colour	No change in colour
2	Voges-Proskauer test	Pink-red colour	No change in colour
3	Triple Sugar Iron slant test	Yellow slant and yellow butt along with gas production	No change or red colour appearance
4	Motility, Indole, Lysine test (MIL)		Diffused growth, red colour on top of tubes, purple colour throughout the medium. Growth along stabbed line, No red colour, yellow colour throughout the medium.
5	Nitrate	Red colour	No change in colour

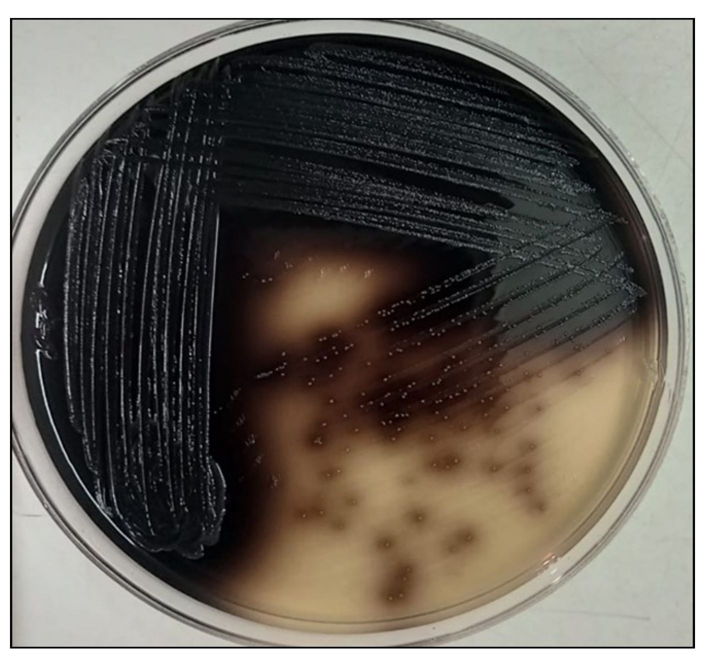

Fig. 4.1: Small black coloured colonies of *Enterococcus* on BEA agar

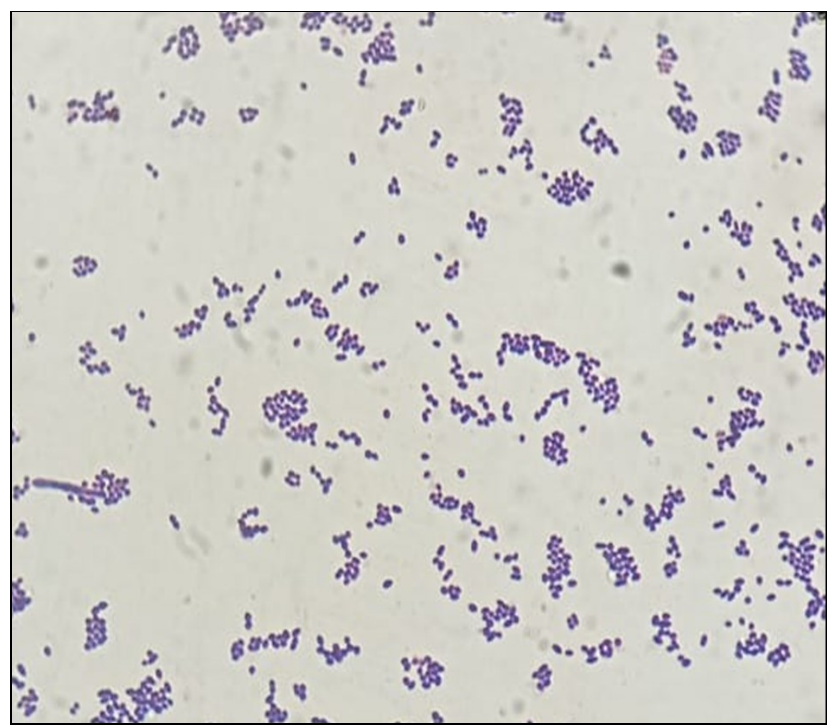

Fig. 4.2: Gram positive cocci of *Enterococcus* under light microscope (100x)

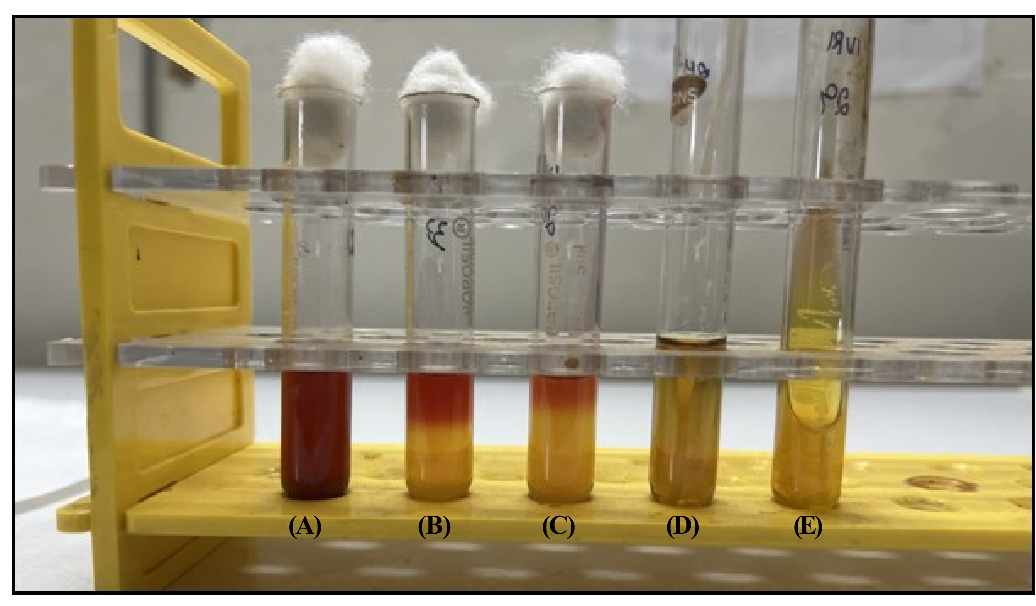

Fig. 4.3: **Biochemical tests for Enterococcus-A-Nitrate, B-Methyl Red, C-Voges Proskeur, D-MIL (Motility, Indole, Lysine), E-TSI (Triple Sugar Iron).**

Table 4.3 Biochemical characterization of *Enterococcus*

TEST	RESULTS
Catalase	Negative
Oxidase	Negative
Methyl Red	Positive
Voges proskeur	Positive
Motility	Negative
Indole Production	Negative
Lysine decarboxylation	Negative
Triple sugar iron test	Positive
Nitratase	Positive
H, S Production	Negative

4.2 Molecular confirmation of purified *Enterococcus* isolates by PCR

After the identification of the *Enterococcus* isolates via primary and secondary biochemical testing, the isolates were subjected to PCR reaction targeting *Sod A* genes. The desired amplicon size of 215 bp and 360 bp were obtained for *E. faecium* and *E. faecalis* genes respectively (Fig 4.4). The positive control was selected after confirmation from Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS).

4.3 Phenotypic characterization for virulence

4.3.1 Biofilm formation by *Enterococcus* isolates

In the study, 11 (8.46%), 15 (11.54%), and 25 (19.23%) isolates of *Enterococcus* had strong, moderate and weak ability to form biofilm, respectively. The description of biofilm forming ability are given in Tables 4.4& 4.5, Fig 4.5.

Table 4.4: Species wise distribution of biofilm forming ability of *Enterococcus* isolates

Species	Strong	Moderate	Weak
E. faecium	2	8	9
E. faecalis	9	7	16
Total	**11**	**15**	**25**

Table 4.5: Source wise distribution of biofilm forming ability of *Enterococcus* isolates

Source of isolates	Strong	Moderate	Weak
Animal faecal swabs	4	4	11
Environment (Water, Soil, Feed, Sewage)	1	5	7
Pork	1	3	3
Human hand swabs	5	3	4
Total	**11**	**15**	**25**

4.3.2 DNase activity by *Enterococcus* isolates

In the study, 27 (20.76%) isolates of *Enterococcus* were showing DNase activity out of which *E. faecium* were 15 (22.38%) and *E. faecalis* were 12 (19.04%). The description of DNase activity is given in Table 4.6 & Fig 4.6.

Table 4.6: Source wise distribution of DNase activity of *Enterococcus* isolates

Source of isolates	DNase activity
Animal faecal swabs	13
Environment (Water, Soil, Feed, Sewage)	7
Pork	2
Human hand swabs	5
Total	27

4.3.3 Hemolytic activity by *Enterococcus* isolates

In our study, hemolytic activity was observed among 8 (12.7%) *E. faecalis* isolates and 17 (25.3%) *E. faecium* isolates and the phenotype was of partial hemolysis (alpha-hemolysis) (Fig 4.7).

4.4 Occurrence of *E. faecium* and *E. faecalis* isolates

On the basis of molecular characterization based on *sodA* gene, a total of 130 isolates were confirmed as *Enterococcus* species out of which 67 were *E. faecium* and 63 were *E.*

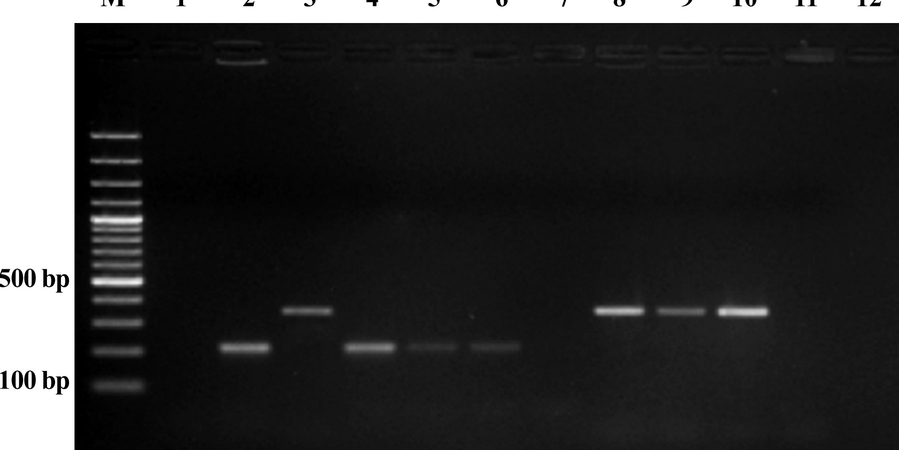

Fig. 4.4: Molecular confirmation of *E. faecium* and *E. faecalis*

Lane M : 100 plus ladder
Lane 1 : Negative control
Lane 2 : Positive control (*E. faecium*)-215bp
Lane 3 : Positive control (*E. faecalis*) -360bp
Lanes 4-6 : Positive strains (*E. faecium*)-215bp
Lanes 8-10 : Positive strains (*E. faecalis*)-360bp

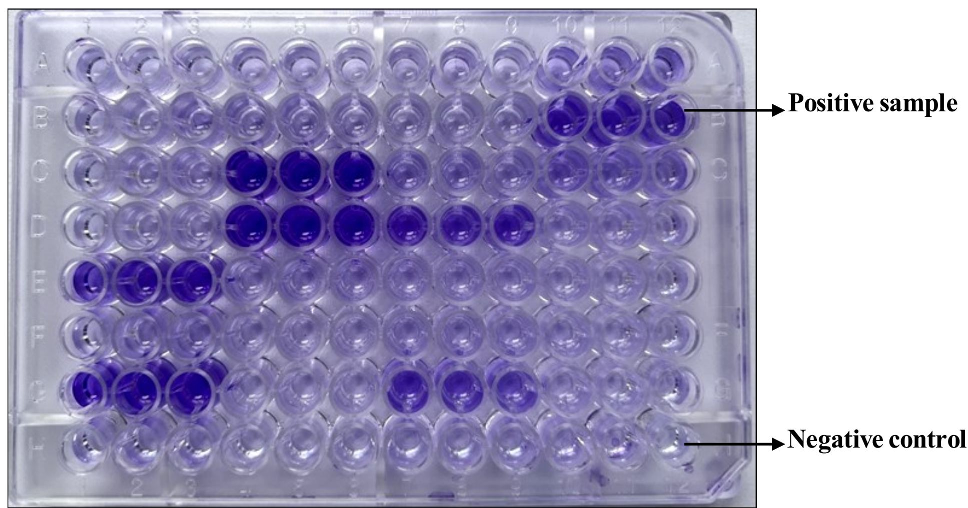

Fig. 4.5: Biofilm forming ability of *Enterococcus* isolates

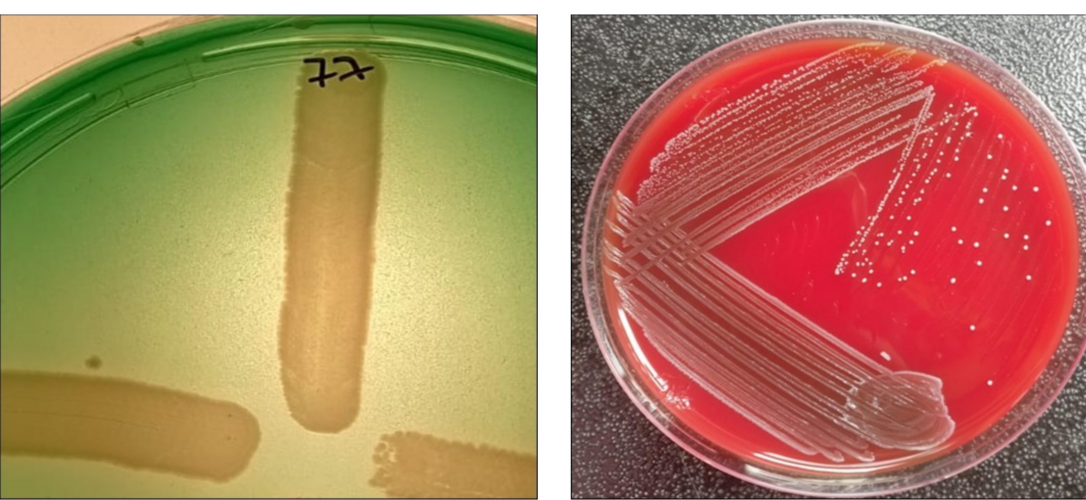

Fig. 4.6: DNase activity of *Enterococcus*

Fig. 4.7: Alpha- hemolysis

faecalis and further tested for antimicrobial susceptibility testing. Out of 130 *Enterococcus* isolates, 67 were isolated from animal faecal swabs, 26 from environmental (Water, Soil, Feed, Sewage) samples, 17 from human hand swabs and 20 from pork. The table 4.4 depicts the Occurrence of *E. faecium* and *E. faecalis* isolates.

Table 4.7: Occurrence of *E. faecium* and *E. faecalis* isolates

Type of Sample	No: of samples			*E. faecium*			*E. faecalis*		
	Bareilly	Krishna	Total	Bareilly	Krishna	Total	Bareilly	Krishna	Total
Faecal swabs	75	50	125	20	22	42	12	13	25
Environment	30	20	50	8	7	15	8	3	11
Human hand swabs	20	5	25	7	3	10	7	0	7
Pork	50	0	50	0	0	0	20	0	20
Total	**175**	**75**	**250**	**35**	**32**	**67/250 (26.8%)**	**47**	**16**	**63/250 (25.2%)**

4.4.1 Place wise isolation

Out of 130 enterococci isolates, 82 (82/175=46.9%) were isolated from Bareilly district of UP of which 35 were *E. faecium* and 47 were *E. faecalis*. From Krishna district of AP 48 (48/75=64%) were isolated of which 32 were *E. faecium* and 16 were *E. faecalis*.

4.4.2 Source wise isolation

Out of 130 enterococci isolates, 67 were isolated from pig faecal swabs, 26 were isolated from environmental (Water-6, Soil-2, Feed-13, Sewage-5) samples, 17 from human hand swabs and 20 from pork samples. Out of 67 *E. faecium* isolates, 42 were from faecal swabs, 15 from environment samples and 10 from human hand swabs. Out of 63 *E. faecalis* isolates, 25 were from faecal swabs, 11 were from environmental samples, 7 were from human hand swabs and 20 were from pork.

4.4.3 Sex and age wise isolation

Out of 67 isolates from animal faecal swabs, 41 were isolated from females and 26 were isolated from males. 8 were isolated from <3 months of age, 27 were isolated from 3-6 months of age and 32 were isolated from > 6 months of age.

4.4.4 Farm wise isolation

Out of 130 isolates, 48 were isolated from three farms of Krishna district of AP (Table 4.8) and 62 were isolated from five farms of Bareilly district of UP (Table 4.9). Twenty isolates from pork which were collected from retail meat shops in Bareilly district of UP.

Table 4.8: Farm wise isolation in Krishna district of AP

Krishna district (AP)	Faecal swabs	Environment (feed,water, soil, sewage)	Human hand swabs	Total
Farm-1	15	4	1	20
Farm-2	11	3	1	15
Farm-3	9	3	1	13
Total	**35**	**10**	**3**	**48**

Table 4.9: Farm wise isolation in Bareilly district of UP

Bareilly district (AP)	Faecal swabs	Environment (feed,water, soil, sewage)	Human hand swabs	Total
Farm-1	10	4	4	18
Farm-2	7	4	3	14
Farm-3	6	3	2	11
Farm-4	6	3	3	12
Farm-5	3	2	2	7
Total	**32**	**16**	**14**	**62**

4.5 Assay for antimicrobial drug resistance of the *E. faecium* and *E. faecalis isolates*

The *Enterococcus* isolates were subjected to *in vitro* antibiotic sensitivity test by disc diffusion method (CLSI, 2020) on Muller-Hinton agar (MHA) plates (Bauer *et al.,* 1966). They were tested against commonly used antibiotics viz. ampicillin (AMP, 10μg), amoxycillin (AMX, 30μg), pencillin-G (P, 10 Units), oxacillin (Ox, 5μg), nitrofurantoin (NIT, 300μg), teicoplanin (TEI, 30μg), ciprofloxacin (CIP, 5μg), linezolid (LZ, 30μg), streptomycin (HLS,

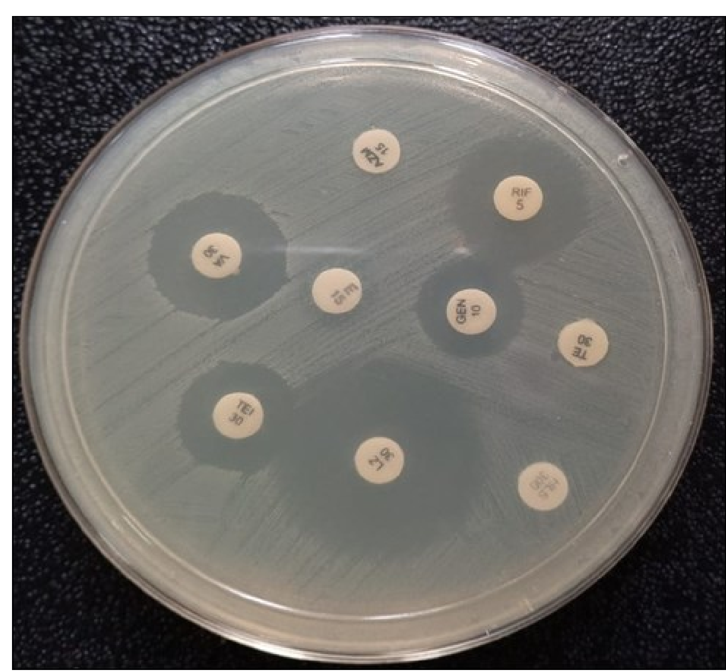

Fig. 4.8: Disc diffusion assay

300µg), azithromycin (AZM, 15µg), rifampin (RIF, 5µg), vancomycin (V, 30µg), erythromycin (E, 15µg), gentamicin (GEN, 10µg) and tetracycline (TE, 30µg).

4.5.1 *Enterococcus isolates* resistant to antimicrobial drugs

The per cent of *Enterococcus* isolates resistant to various antimicrobial drugs by disc diffusion method are shown in table 4.10. Among 130 isolates of *E. faecium* and *E. faecalis* tested for resistance against various antibiotics and isolates were found to be resistant to the following antibiotics oxacillin (100%), rifampicin (67.7%), azithromycin (50.8%), tetracycline (47.75), erythromycin (43.1%), gentamicin (38.5%), ciprofloxacin (21.5%), high level streptomycin (14.6%), pencillin (12.3%), linezolid (8.5%), nitrofurantoin (6.2%) and teicoplanin (3.8%).

However, among the isolates tested were found to be intermediate to erythromycin (27.7%), ciprofloxacin (23.1%), gentamicin (17.7%), rifampin (10%), linezolid (9.2%), teicoplanin (4.6%), nitrofurantoin (3.8%) and vancomycin (2.3%).

Table 4.10: *Enterococcus* isolates resistant, intermediate and sensitive to antimicrobial drugs

S. No.	Antimicrobial agents	Sensitive (%)	Intermediate (%)	Resistant (%)
I.	**Penicillins**			
1	Ampicillin (AMP)	100	0	0
2	Amoxicillin (AMX)	100	0	0
3	Pencillin(P)	87.7	0	12.3
4	Oxacillin(O)	0	0	100
II.	**Glycopeptides**			
5	Vancomycin (V)	97.7	2.3	0
III.	**Lipoglycopeptides**			
6	Teicoplanin (TEI)	91.5	4.7	3.8
IV.	**Aminoglycosides**			
7	Gentamicin (GEN)	43.8	17.7	38.5
8	Streptomycin (HLS)	85.4	0	14.6
V.	**Tetracyclines**			
9	Tetracycline (TET)	52.3	0	47.7
VI.	**Fluoroquinolones**			
10	Ciprofloxacin (CIP)	55.4	23.1	21.5

VII.	**Nitrofurantoins**			
11	Nitrofurantoin (N)	90.0%	3.8%	6.2%
VIII.	**Ansamycins**			
12	Rifampin (RIF)	22.3%	10%	67.7%
IX.	**Macrolides**			
13	Azithromycin (AZM)	49.2%	-	50.8%
14	Erythromycin(E)	29.2%	27.7%	43.1%
X.	**Oxazolidinones**			
15	Linezolid (L)	82.3%	9.2%	8.5%

4.6 Chi square test for antibiotic resistance pattern with different epidemiological factors

4.6.1.1 Place-wise resistance pattern of enterococci isolates

Out of 130 enterococci isolates, 82 were isolated from Bareilly (UP), 48 from Krishna (AP) and these were tested for antimicrobial susceptibility assay and the results are shown (Table 4.11 and Fig 4.9). Erythromycin resistance in Bareilly district of UP was significantly (p, 0.04) higher than in Krishna district of AP. Nitrofurantoin resistance in Krishna district of AP was significantly (p, 0.03) higher than in Bareilly district of UP.

Table 4.11: Place-wise antibiotic resistance pattern of *Enterococcus* isolates

Antibiotic disc	Resistance percentage (%)		p value
	Bareilly (UP)	Krishna (AP)	
Vancomycin	0	0	>0.05
Erythromycin	56	10	0.04
Gentamicin	37.5	40	>0.05
Tetracycline	56.2	30	>0.05
Ampicillin	0	0	NA
Amoxicillin	0	0	NA
Penicillin	12.5	10	>0.05
Oxacillin	100	100	NA
Teicoplanin	0	15	>0.05
Ciprofloxacin	18.8	40	>0.05
Linezolid	6.3	0	>0.05
High-level streptomycin	18.7	30	>0.05
Azithromycin	68.2	50	>0.05
Rifampicin	75	70	>0.05
Nitrofurantoin	6.2	10	0.03

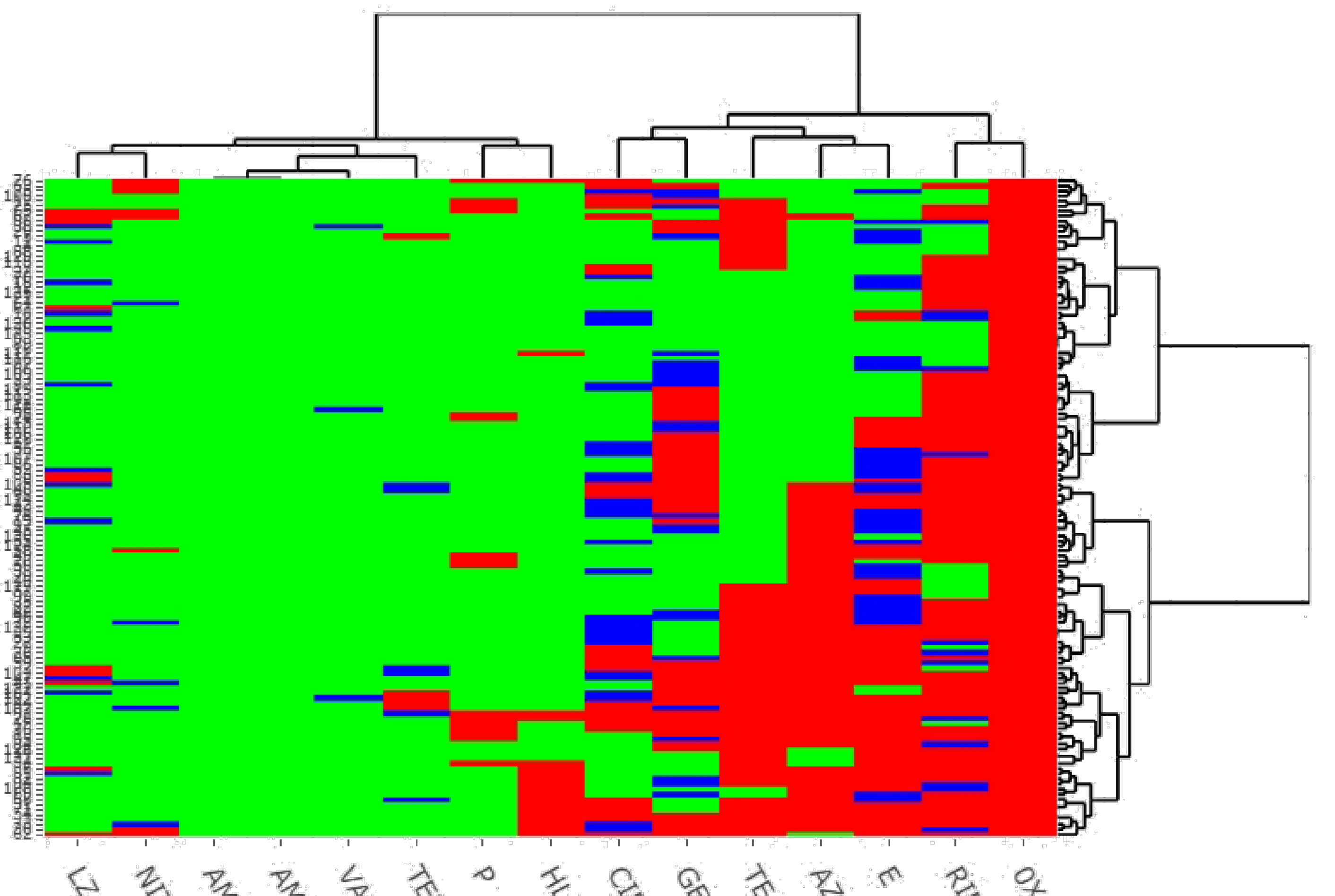

Fig. 4.9: **Cluster heat map of *E.faecium* and *E.faecalis* isolates showing isolate based clustering in row and antibiotic resistance based clustering on column.**

Red colour indicates resistance, blue colour indicates intermediate resistance and green colour indicates susceptible.

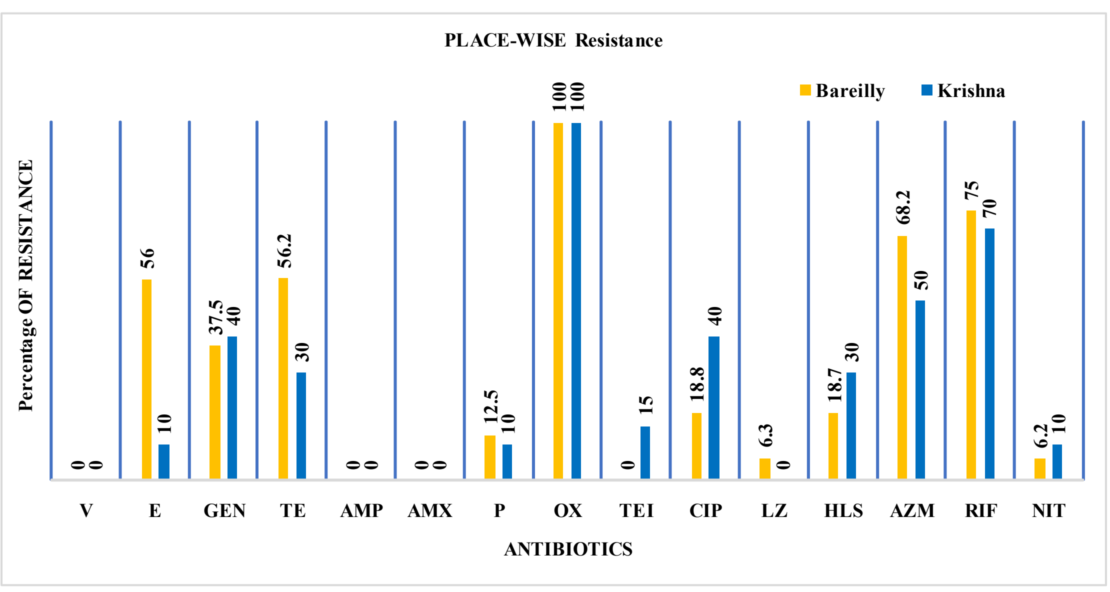

Fig. 4.10: Place wise resistance pattern

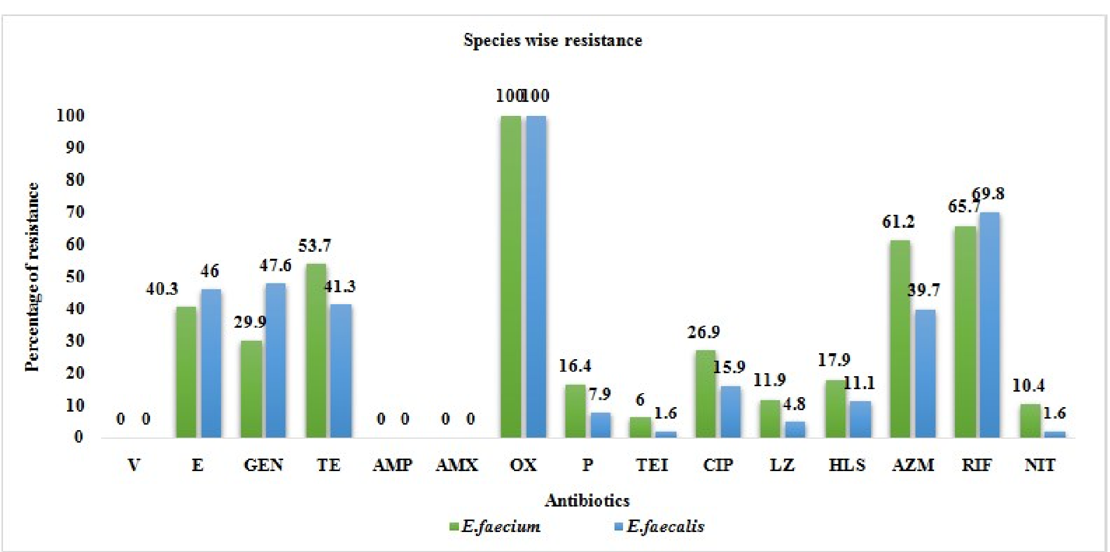

Fig. 4.11: Species wise resistance pattern

4.6.1.2 Species-wise antibiotic resistance pattern of enterococci isolates

Out of 130 enterococci isolates, 67 isolates were *E. faecium*, 63 isolates were *E. faecalis* and their resistance patterns are shown (Table 4.12 and Fig 4.10). Gentamicin resistance in *E. faecalis* isolates were significantly (p, 0.04) higher than *E. faecium*. Azithromycin resistance in *E. faecium* were significantly (p, 0.01) higher than *E. faecalis*. Nitrofurantoin resistance in *E. faecium* were significantly (p, 0.007) higher than *E. faecalis*.

Table 4.12: Species-wise antibiotic resistance pattern of *Enterococcus* isolates

Antibiotic disc	Resistance percentage (%)		p value
	E. faecium	*E. faecalis*	
Vancomycin	0	0	NA
Erythromycin	40.3	46	>0.05
Gentamicin	29.9	47.6	0.04
Tetracycline	53.7	41.3	>0.05
Ampicillin	0	0	NA
Amoxicillin	0	0	NA
Pencillin	16.4	7.9	>0.05
Oxacillin	100	100	NA
Teicoplanin	6	1.6	>0.05
Ciprofloxacin	26.9	15.9	>0.05
Linezolid	11.9	4.8	>0.05
High-level streptomycin	17.9	11.1	>0.05
Azithromycin	61.2	39.7	0.01
Rifampin	65.7	69.8	>0.05
Nitrofurantoin	10.4	1.6	0.007

4.6.1.3 Source-wise antibiotic resistance pattern of enterococci isolates

Out of 130 enterococci isolates, 67 isolated were from animal faecal swabs,26 isolated were from environment (Water, Soil, Feed, Sewage), 20 isolated were from pork,17 isolated were from humans and their resistance patterns are shown (Table 4.13 and Fig 4.11). Erythromycin resistance (p, 0.01), penicillin resistance (p, 0.04), ciprofloxacin resistance (p, 0.02), azithromycin resistance (p, 0.001) in enterococci from human hand swabs were significantly higher than from other sources of samples. Tetracycline resistance (p, 0.03), and

linezolid resistance (p, 0.02) in enterococci from animal faecal swabs were significantly higher than from other sources of samples.

Table 4.13: Source wise antibiotic resistance pattern of *Enterococcus* isolates

Antibiotic disc	Resistance (%)				
	Faecal swabs	Environment	Human hand swabs	Pork	p value
V	0	0	0	0	NA
E	43.3	38.5	70.6	25	0.01
GEN	38.8	38.5	41.2	35	>0.05
TE	56.7	46.2	47.1	20	0.03
AMP	0	0	05	0	NA
AMX	0	0	0	0	NA
P	11.9	11.5	29.4	0	0.04
OX	100	100	100	100	NA
TEI	4.5	7.7	0	0	>0.05
CIP	20.9	26.9	35.3	5	0.02
LZ	14.9	3.8	0	0	0.02
HLS	13.1	23.1	17.6	5	>0.05
AZM	55.2	61.5	64.7	10	0.001
RIF	61.2	73.1	76.45	75	>0.05
NIT	7.5	7.7	5.9	0	>0.05

4.6.1.4 Sex-wise antibiotic resistance pattern of enterococci isolates

Out of 67 enterococci isolates from animal faeces, 26 isolated were from males, 41 isolated were from females and their resistance patterns are shown (Table 4.14 and Fig 4.12).

Table 4.14: Sex-wise antibiotic resistance pattern of *Enterococcus* isolates

Antibiotic disc	Resistance percentage (%)		p value
	Male	Female	
Vancomycin	0	0	NA
Erythromycin	43.9	42.3	>0.05
Gentamicin	29.3	53.8	>0.05
Tetracycline	58.5	53.8	>0.05
Ampicillin	0	0	NA
Amoxicillin	0	0	NA
Pencillin	4.9	23.1	>0.05

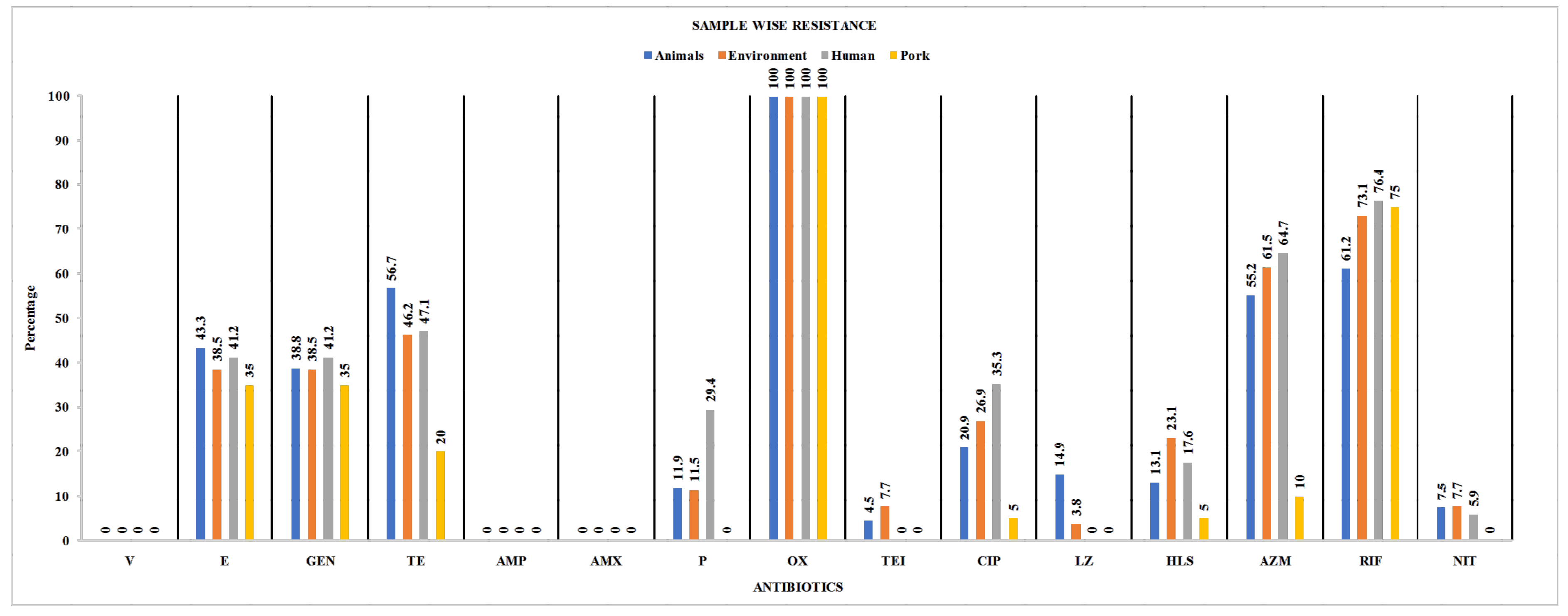

Fig. 4.12: Sample wise resistance pattern

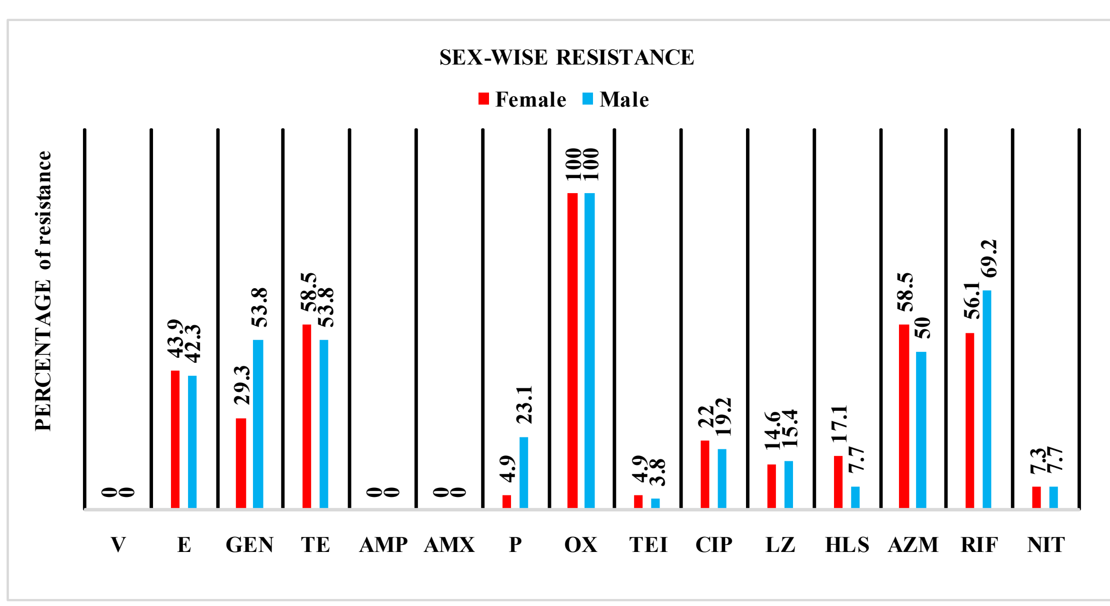

Fig. 4.13: Sex wise resistance pattern

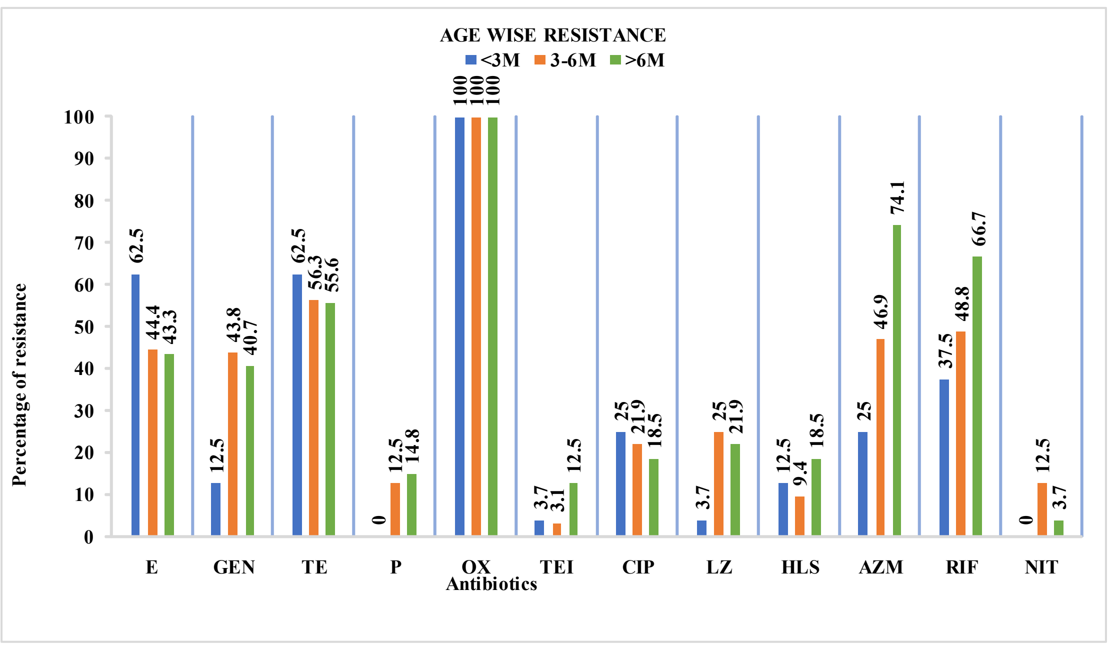

Fig. 4.14: Age wise resistance pattern

Oxacillin	100	100	NA
Teicoplanin	4.9	3.8	>0.05
Ciprofloxacin	22	19.2	>0.05
Linezoild	14.6	15.4	>0.05
High-level streptomycin	17.1	7.7	>0.05
Azithromycin	58.5	50	>0.05
Rifampin	56.1	69.2	>0.05
Nitrofurantoin	7.3	7.7	>0.05

4.6.1.5 Age-wise antibiotic resistance pattern of enterococci isolates

Out of 67 enterococci isolates from animal faeces, 8 isolated were from animal aged less than 3 months ,27 isolated were from animals aged between 3-6 months, 32 isolated were from animals aged more than 6 months and their resistance patterns are shown (Table 4.15 and Fig 4.13). Linezolid resistance in enterococci from animals aged 3-6 months was significantly (p, 0.03) higher than <3 months and >6 months age groups. Azithromycin resistance from animals aged above 6 months was significantly (p, 0.02) higher than <3 months and 3-6 months age groups.

Table 4.15: Age- wise antibiotic resistance pattern of *Enterococcus* isolates

Antibiotic disc	Resistance percentage (%)			p value
	<3M	3-6M	>6M	
Vancomycin	0	0	0	NA
Erythromycin	62.5	44.4	43.3	>0.05
Gentamicin	12.5	43.8	40.7	>0.05
Tetracycline	62.5	56.3	55.6	>0.05
Ampicillin	0	0	0	NA
Amoxicillin	0	0	0	NA
Pencillin	0	12.5	14.8	>0.05
Oxacillin	100	100	100	NA
Teicoplanin	3.7	3.1	12.5	>0.05
Ciprofloxacin	25	21.9	18.5	>0.05
Linezolid	3.7	25	21.9	0.03
High-level streptomycin	12.5	9.4	18.5	>0.05
Azithromycin	25	46.9	74.1	0.02
Rifampin	37.5	48.8	66.7	>0.05
Nitrofurantoin	0	12.5	3.7	>0.05

4.7 Multiple Drug Resistant *Enterococcus*

Enterococci isolates which were resistant to at least three drugs were designated as multiple drug resistant (MDR) isolates. In total, 50 (38.46%) enterococci isolates were MDR type of which 27 (20.76%) are *E. faecium* and 23 (17.69%) are *E. faecalis*.

4.8 Phenotypic detection of VRE by disc diffusion method

All 130 isolates of enterococci were subjected to screening by disc diffusion method using vancomycin (30 µg) drug. A total of 3 (2.3%) isolates were showing intermediate resistant to vancomycin by disc diffusion method. All 3 isolates showed MIC of>4µg/ml for vancomycin as per CLSI guidelines (Table 4.16).

Table 4.16: VRE occurrence

VRE	Place	Species	Interpretation
Human hand swab	Bareilly (UP)	*E.faecalis*	Intermediate
Animal faecal swab	Bareilly (UP)	*E.faecalis*	Intermediate
Animal faecal swab	Krishna (AP)	*E.faecalis*	Intermediate

4.9 Molecular confirmation of VRE

The DNA was extracted using Snap chill method from all 130 enterococci isolates confirmed by phenotypic methods. The extracted DNA was used as template for PCR reactions for genotypic detection and confirmation of vancomycin producing genes (*vanA*, *vanB*, *vanC1)*. Only 2 *E. faecalis* isolates (1.5%) were positive with PCR with amplicon band at 402 bp for *vanC1* (Fig 4.15). None of the isolates were positive for *vanA* and *vanB* genes.

4.9.1 Sanger sequencing and data analysis

The two isolates of *E. faecalis* (one from animal faecal swab and other from human hand swab and both were from two different pig farms in Bareilly district of UP) which were showing presence of *vanC1* gene were chosen for sequencing and sent to Barcode Biosciences, Bangalore.The data then assembled using MEGA X software. The final contigs of each gene were blasted in NCBI database. BLAST of the sequence matched 99 to 100% with globally

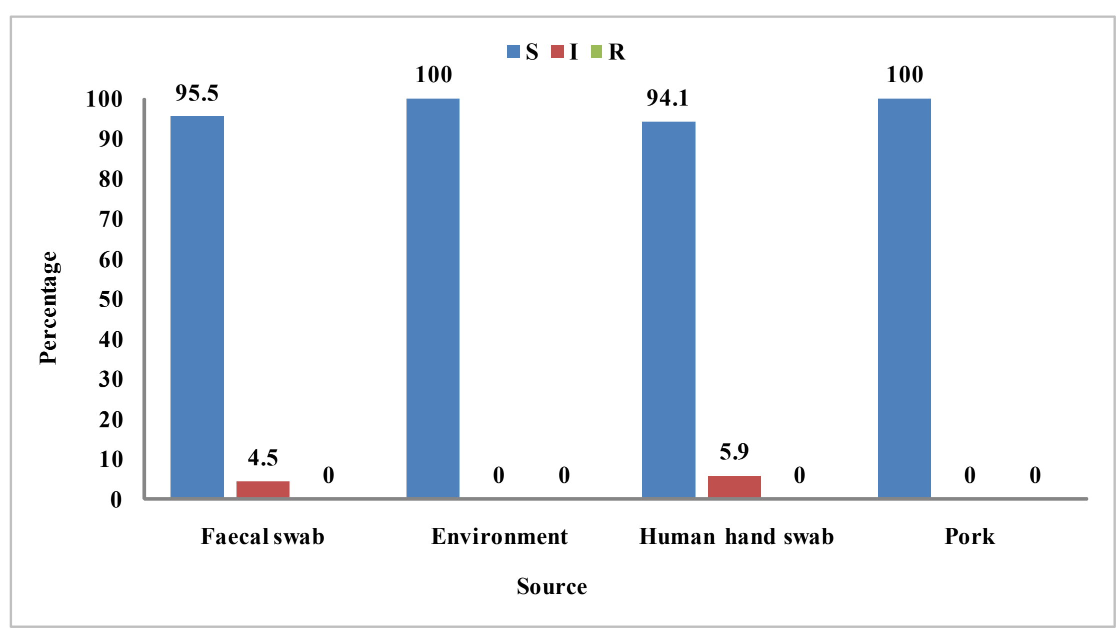

Fig. 4.15: Source wise intermediate resistance to vancomycin

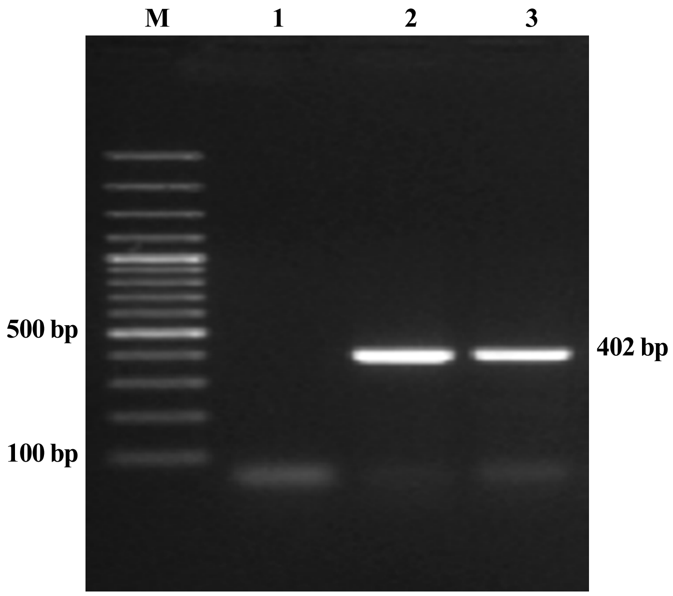

Fig. 4.16: Molecular confirmation of *vanC1* gene

Lane L : 100 plus ladder

Lane 1 : Negative control

Lane 2-3 : Positive samples (*vanC1* gene)-402 bp

submitted *E. faecalis* with *vanC1* gene strains isolated from sheep milk, poultry internal organs and calf diarrhoea..

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Discussion

5 DISCUSSION

The fight against nosocomial infections caused by multidrug-resistant bacteria of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species) group is one of the most serious challenges in modern medicine (Santajit and Indrawattana, 2016; Mulani *et al*., 2019; Ma *et al*; 2020). Due to the potential for the emergence of novel and complex drug resistance mechanisms, these pathogens pose a serious threat. The phrase "escape" of these bacteria from antibiotic action is exactly what the acronym ESKAPE alludes to (Ma *et al*., 2020). Hospitalized, immunocompromised and patients with intestinal flora dysbiosis are particularly susceptible to these bacterial infections, which constitute a major threat to their lives and health (Alagna *et al*., 2020; Nasser *et al*., 2020). Lot of research is going on ESKAPE bacteria in medical sciences, but little is understood about their occurrence in animals and its relevance to health of livestock owners and veterinarians (Singh, 2017).

In addition to inhabiting the gastrointestinal tracts of humans, other mammals, birds, reptiles, and insects, enterococci are gram-positive facultative anaerobes that can also be found in soil, water, and food (Miller *et al*., 2014; Lebreton *et al*., 2014). On the other hand, enterococci are opportunistic human infections that frequently develop resistance to a wide range of antibiotics. Normally harmless for healthy people, enterococci can cause in endocarditis, bacteremia, infections of the urinary tract, wounds, intra-abdominal and pelvic areas, and superinfections in immunocompromised people (Love, 2001; Ramos *et al*., 2020). With a mortality rate of up to 23%, enterococci are second to staphylococci as the most common cause of nosocomial infections (Bhardwaj, 2019). There are more than 50 species in the

genus *Enterococcus*, however *Enterococcus faecalis* and *Enterococcus faecium* make up more than 80% of human clinical isolates (Torres *et al*., 2018) and are among the most prevalent bacteria in both humans and animals gut microbiota (Lebreton *et al*., 2014).

Fecal enterococci can contaminate food of animal's origin at slaughter houses. Some of the studies reported that *E. faecalis* and *E. faecium* are the enterococci that are found in over 90% of food samples of animal origin (Boehm and Sassoubre, 2014; Tyson *et al*., 2018). It has been established that these two species can be found in processed and uncooked foods such as cheese, fish, sausages, minced beef, pig, and ready-to-eat items (Ghosh and Zurek, 2015). Infections in food animals and poultry have also been linked to *E. faecalis* and *E. faecium* (Tankson *et al*., 2001; Steentjes *et al*., 2002; Wyder *et al*., 2011). *E. faecium* and *E. faecalis* are the 2 species most frequently associated with a range of enterococcal diseases in clinical settings, which account for one-third of whole nosocomial infections through the world (Miller *et al*., 2014; Weigel *et al*., 2003).

It is currently projected that the use of antibiotics in food animals will rise by 67% by 2030, two-thirds of which is expected to be used in intensive food animal production, with the use in pig and poultry production expected to double (Van Boeckel *et al*., 2015). Therefore, it is crucial to understand how this could affect the spread of resistant bacteria through the food chain by studying the distribution of these species along entire food production chains. The spread of MDR strains can occur through direct (consumption of contaminated food, direct contact of farmers and veterinarians) or indirect (animal waste handling, contaminated ground water or surfaces) routes (Daniel *et al*., 2015). The resistance determinants carried by the MDR strains could also be transmitted to the other commensal strains in the host and cause further complications (Price *et al*., 2019). In addition, infections caused by MDR strains have been associated with long hospital stays, high morbidity and mortality rate (Beganovic *et al*., 2018).

Previous studies have confirmed that multi-drug resistant enterococci from animal sources can donate their resistant genes intraspecific and interspecific, and the risk of infections in humans may soon be a reality (Klare *et al*., 1995; Hammerum *et al*., 2017). Many literatures

revealed that there is an increase in resistance especially vancomycin in enterococcus isolated from different species of animals and their products as well as well as humans.

Considering above facts, the present study was carried out to isolate and characterize the enterococci isolated from pigs, pork and their environment (Feed, water, soil and sewage). Thereafter to identify the resistance to commonly used antibiotics especially vancomycin among enterococci isolates and genotypically detect vancomycin encoding genes (*vanA*, vanB and *vanC1*) in them. Furthermore, to determine the association of various epidemiological factors associated with enterococci isolates collected from different pig farms located in Bareilly district of UP and Krishna district of AP were carried out.

The biochemical and morphological characteristics of the *Enterococcus* isolates in the study were consistent with the description of typical enterococci described by Beshiru *et al.* (2017) and Ramos *et al.* (2020) and as a group of Gram-positive cocci, non-spore forming, catalase negative organisms. All of the isolates had the required characteristics to qualify for the genus. The cell characteristics of all the isolates were also consistent with those of classical enterococci.

In the present study, overall isolation of *Enterococcus* spp. (64%) was almost in agreement with the findings of Giao *et al.* (2022) who reported an overall isolation rate of 62% in swine samples and Thu *et al.* (2019) who reported an overall isolation was 75% in a sampling frame containing 648 faecal samples whereas higher the isolation rate of 99% was reported by Hayes *et al.* (2003), with a sample frame containing 981 samples. The difference in isolation rate of *Enterococcus* in the present study may be due to difference in geographic location and rearing practices.

Among the 160 *Enterococcus* isolates, the multiplex PCR assays identified 42% of the isolates as *E. faecium* (n=67) and 40% of isolates as *E. faecalis* (n=63) and both the species representing about 81% (130) of the total isolates. The observations were in almost agreement with Giao *et al.* (2022) who reported 90% of the isolates including *E. faecium*, *E. faecalis* and *E. hirae*. Among 67 isolates of *E. faecium*, 42 were isolated from faecal swabs, 15 from environment (Feed, water, soil and sewage) samples, 10 from human hand swabs.

Among 63 isolates of *E. faecalis*, 25 were isolated from faecal swabs, 11 from environment (Feed, water, soil and sewage) samples, 7 from human hand swabs and 20 from pork samples.

All 130 isolates were tested for biofilm formation, DNase activity and hemolytic activity. In the study, 11 (8.46%), 15 (11.54%), and 25 (19.23%) isolates of *Enterococcus* had strong, moderate and weak ability to form biofilm, respectively. This study showed that *E. faecalis* has a higher potential to form biofilm than *E. faecium*. Several workers have also reported that *E. faecalis* had a higher potential to form biofilm than *E. faecium* (Tan *et al*., 2018; Cui *et al*., 2020). In our study, hemolytic activity was observed among 8(12.7%) *E. faecalis* isolates and 17(25.3%) *E. faecium* isolates and the phenotype was of partial hemolysis (alpha-hemolysis). Hemolytic activity was more among *E. faecium* isolates than *E. faecalis* isolates. Rotta *et al*. (2022) also reported that hemolytic activity was more among *E. faecium* than *E. faecalis*. In the study, 27 (20.76%) isolates of *Enterococcus* were showing DNase activity out of which *E. faecium* were 15 (22.38%) and *E. faecalis* were 12 (19.04%). In our study DNase activity predominated among *E. faecium* isolates, in contrast to Rotta *et al*. (2022) who reported DNase activity predominant among *E. faecalis* isolates.

In the present study *in vitro* antibiotic resistance pattern of the isolates were determined by disc diffusion method. Antibiotic susceptibility assay of 130 enterococci isolates revealed that all isolates (100%) were sensitive to ampicillin. The observations corroborated with previous studies on the resistance of *Enterococcus faecalis* and *Enterococcus faecium* isolated from food-producing animal samples in Russia (Makarov *et al*., 2022). The present study depicted 39%, 43% and 48% resistance levels towards gentamicin, erythromycin and tetracycline respectively. Almost similar results of 33%, 41% and 43% resistance towards gentamicin, erythromycin and tetracycline respectively were obtained by Zhao *et al*. (2022) on a study of tracing enterococci persistence along a pork production chain from feed to food in China. Igbinosa *et al*. (2020) reported 47% and 24% resistance towards tetracycline and ciprofloxacin respectively which were almost similar to the present study showing 48% and 22% resistance towards tetracycline and ciprofloxacin respectively. The present study depicted 68% resistance levels towards rifampicin. Almost similar results of 64% resistance towards rifampicin was reported by Cui *et al*. (2020).

Antibiotic susceptibility assay of 130 enterococci isolates revealed that all isolates (100%) were resistant to oxacillin. Almost similar results of 95% resistance towards oxacillin was obtained by Citak *et al*. (2005). The present study showed 6% resistance towards nitrofurantoin which was almost similar to the findings observed by Badul *et a*l. (2021) who reported 3.2% resistance towards nitrofurantoin on a study of antibiotic resistant *Enterococcus* spp in intensive pig farming in South Africa. The present study depicted 14.6% resistance level towards high level streptomycin and it showed lower range compared to 20.4% reported by de campos *et al*. (2015).

In present study, species-wise antibiotic susceptibility pattern of enterococci showed that *E. faecium* had higher significance for acquiring antibiotic resistance than *E. faecalis* against the antibiotics viz, tetracycline, pencillin, teicoplanin, ciprofloxacin, linezolid, high level streptomycin, azithromycin and nitrofurantoin. Several workers have also reported that *E. faecium* had higher significance for acquiring antibiotic resistance than *E. faecalis* (Azizi *et al*., 2022; Makarov *et al*., 2022; Rajendiran *et al*., 2022).

Place-wise antibiotic susceptibility pattern of enterococci showed that enterococci isolates from Krishna district of Andhra Pradesh (AP) had higher significance for acquiring antibiotic resistance against the antibiotics viz, tetracycline, teicoplanin, ciprofloxacin, linezolid, high level streptomycin, azithromycin and nitrofurantoin than isolates from Bareilly district of Uttar Pradesh (UP). This may be due to higher use of antibiotics in Krishna district of AP farms either for treatment or as prophylaxis. Enterococci isolates from Bareilly district of UP had higher significance for acquiring erythromycin resistant isolates than that of enterococci from Krishna district of AP. To compare the findings, there were no earlier reports available on comparison of pattern of antibiograms of enterococci strains from different regions in India.

In present study, source-wise antibiotic susceptibility pattern of enterococci showed that enterococci isolates from human hand swabs had higher significance for acquiring antibiotic resistance against the antibiotics viz, erythromycin, gentamicin, ciprofloxacin, linezolid, pencillin, azithromycin and rifampin than isolates from animal faecal swabs, environment and pork samples. This may be due to higher use of antibiotics by humans for treatment. Enterococci isolates

from animal faeces had higher significance for acquiring tetracycline, linezolid resistance than that of enterococci from other sources. Enterococci isolates from environment had higher significance for acquiring teicoplanin and high-level streptomycin resistance than that of enterococci isolates from other sources. Tan *et al*. (2018) reported different resistance patterns of *Enterococcus faecalis* and *Enterococcus faecium* in the pigs, farmers and farm environments in Malaysia.

The antibiotic susceptibility pattern of enterococci isolates from male and female pigs showed no statistical association with respect to acquiring of antibiotic resistance. Therefore sex of pigs has no impact on enterococci isolates and are equally prone for acquiring antibiotic resistance.

Antibiotic susceptibility assay of *E. faecalis* revealed 4.8% resistance and *E. faecium* revealed 11.9% resistance to Linezolid. Similar findings of 5.4% in *E. faecalis* was reported but lower in *E. faecium* (2.8%) was reported by Xuan *et al*. (2021) who studied on antimicrobial resistance in *E. faecium* and *E. faecalis* isolates in swine. The present study revealed a resistance level of 15.9% and 26.9% towards Ciprofloxacin in *E. faecalis* and *E. faecium* respectively. A little bit lower results of 9.3% and 15.8% were obtained by Badul *et al*. (2021) on a study of *Enterococcus* species antimicrobial resistance in pig farms

The study was also aimed to detect specific gene (*vanA*, *vanB* and *vanC1*) in isolates which were showing resistance to vancomycin. Three isolates were showing intermediate resistance to vancomycin both by disc diffusion and MIC and when screened for vancomycin resistance genes two isolates of *E. faecalis* were showing *vanC1* gene which is the intrinsic property of *E. gallinarum*. None of the isolates showed *vanA* and *vanB* genes. This peculiar finding of presence of *vanC1* gene in *E. faecalis* is supported by few authors (de Garnica *et al*., 2013; Nishiyama *et al*., 2015; Tatsing and Ateba, 2019). Because the veterinary use of glycopeptide compounds is not permitted in animals, we can presume, as in the case of porcine isolates, that the presence of the *vanC1* gene in *E. faecalis* isolates may be due to the horizontal transfer between *E. gallinarum* and *E. faecalis*. This occurrence of *vanC1* genotype in *E. faecalis* isolates emphasize the need for screening for the presence of both acquired and intrinsic glycopeptide resistance genes.

Based on results of present study, it can be concluded that the samples collected from pigs,pork and associated environment (Soil, Water, sewage and feed) from different farms in Bareilly district (UP) and Krishna district (AP) were not good source of vancomycin resistant enterococci (VRE) which has public health significance.

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Summary and Conclusions

6 SUMMARY AND CONCLUSIONS

The usage of antibiotics, persistence of antibiotic residues, and presence of resistant bacteria in the human–animal–environment niches are associated with the One Health triad due to the interdependence of these pillars in the food chain and environment. The issue of emerging resistant microorganisms associated with livestock is closely linked to improper use of antimicrobial agents in veterinary care, as well as to international trade of food of animal origin, which can contribute to the spread of resistant strains.

The present study was carried out to identify the vancomycin production in enterococci isolates from pigs, pork and their environment and to characterize phenotypically followed by genotypically detection of vancomycin coding genes (*vanA*, *vanB* and *vanC1*). Furthermore, the association of various epidemiological factors with antibiotic susceptibility from enterococci isolates from pig farms collected from Bareilly district of Uttar Pradesh and Krishna district of Andhra Pradesh was determined.

In the present study, a total of 160 enterococci isolates were isolated from 250 samples which included pig faecal, environmental, pork and human hand swabs and were tested for antibiotic resistance. All 160 (64%) isolates were producing black halos on streaking in BEA agar and are further confirmed by biochemical characterization and 130 isolates were found to be *E. faecium*(n=67) and *E. faecalis* (n=63) confirmed by PCR.

All 130 enterococci isolates tested for biofilm formation revealed that, 11 (8.46%), 15 (11.54%), and 25 (19.23%) isolates had strong, moderate and weak ability to form biofilm, respectively. Hemolytic activity was observed among 8(12.7%) *E. faecalis* isolates and

17(25.3%) *E. faecium* isolates and the phenotype was of partial hemolysis (alpha-hemolysis). 27 (20.76%) isolates of *Enterococcus* were showing DNase activity out of which *E. faecium* were 15 (22.38%) and *E. faecalis* were 12 (19.04%).

The antibiotic sensitivity test revealed that enterococci isolates were resistant to oxacillin (100%) most common followed by rifampicin (67.7%), azithromycin (50.8%), tetracycline (47.7%), erythromycin (43.1%), gentamicin (38.5%), ciprofloxacin (21.5%), high level streptomycin (14.6%), pencillin (12.3%), linezolid (8.5%), nitrofurantoin (6.2%), and teicoplanin (3.8%).

Out of 130 enterococci isolates, 45 were isolated from Krishna district of AP, 85 from Bareilly district of UP and these were tested for antimicrobial susceptibility assay. Chi-square test was applied to know the significant difference between antibiotic susceptibility pattern and epidemiological factors. Isolates from Krishna district of AP were showing significantly higher resistance to antibiotics than isolates from Bareilly district of UP. Presence of *vanC1* gene in isolates from Bareilly was higher than the isolates from Krishna.

Out of 130 enterococci isolates, 67 isolates were *E. faecalis* and 63 isolates were *E. faecium* and these were tested for antimicrobial susceptibility assay. Chi-square test was applied to know the significant difference between antibiotic susceptibility pattern. *E. faecium* Isolates showing significantly higher resistance to antibiotics than *E. faecalis* isolates.

Out of 130 enterococci isolates, 67 were isolated from animal faeces, 26 from environment, 20 were isolated from pork, 17 were isolated from human hand swabs and these were tested for antimicrobial susceptibility assay. Chi-square test was applied to know the significant difference between antibiotic susceptibility pattern. Isolates from human hand swabs were showing significantly higher resistance to antibiotics than isolates from other sources.

Out of 67 enterococci isolates from pigs, 26 were from male and 41 from female pigs. There was no significant statistical association observed between the sex of the piglets for antibiotic susceptibility pattern in enterococci isolates. Out of 67 enterococci isolates from pigs, 8 isolates were of <3 months old pigs, 27 from 3-6months old pigs and 32 from >6 months old pigs. Isolates from 3-6 months old pigs were showing significantly higher resistance than isolates from <3 months and >6 months old pigs.

The study was also aimed to detect the molecular regulation of vancomycin resistance in *Enterococcus* through detection of specific genes (*vanA*, *vanB* and *vanC1*) reported earlier in India. The study on molecular detection of *vanA*, *vanB* and *vanC1* genes by PCR amplification among *Enterococcus* isolates showing intermediate resistance towards vancomycin revealed that 2 isolates were harbouring *vanC1* gene and none of the isolates showed *vanA* and *vanB* genes. From the present study it can be concluded that the samples collected from pigs, pork and associated environment (Soil, Water, sewage and feed) from different farms in Bareilly district (UP) and Krishna district (AP) were not good source of vancomycin resistant enterococci (VRE) which has public health significance and the occurrence of *vanC1* genotype in *E. faecalis* isolates emphasize the need for screening for the presence of both acquired and intrinsic glycopeptide resistance genes.

Mini Abstract

7 MINI ABSTRACT

The present study was carried out to identify the vancomycin resistance in enterococci isolates from pigs, pork and their environment and to characterize phenotypically followed by genotypically detection of vancomycin coding genes (*vanA*, *vanB* and *vanC1*) in enterococci isolates. Furthermore, the association of various epidemiological factors with antibiotic susceptibility from enterococci isolates from pig farms collected from Bareilly district of Uttar Pradesh and Krishna district of Andhra Pradesh was determined. In the present study, a total of 160 enterococci isolates were isolated from 250 samples which included pig faecal, environmental, pork and human hand swabs and were tested for antibiotic resistance. All 160 (64%) isolates were producing black halos on streaking in BEA agar and are further confirmed by biochemical characterization and 130 isolates were found to be *E. faecium*(n=67) and *E. faecalis* (n=63) confirmed by PCR. Out of 130 enterococci isolates, 45 were isolated from Krishna district of AP, 85 from Bareilly district of UP. Out of 130 enterococci isolates, 67 were isolated from animal faeces, 26 from environment, 20 were isolated from pork, 17 were isolated from human hand swabs. The antibiotic sensitivity test revealed that all enterococci isolates were resistant to oxacillin (100%) most common followed by rifampicin (67.7%), azithromycin (50.8%), tetracycline (47.7%), erythromycin (43.1%), gentamicin (38.5%), ciprofloxacin (21.5%), high level streptomycin (14.6%), pencillin (12.3%), linezolid (8.5%), nitrofurantoin (6.2%), and teicoplanin (3.8%). Out of 130 isolates, 50 (38.46%) isolates were found to be of Multiple drug resistant type. All 130 enterococci isolates tested for biofilm formation revealed that, 11 (8.46%), 15 (11.54%), and 25 (19.23%) isolates had strong, moderate and weak ability to form biofilm, respectively. All 130 isolates of enterococci were subjected to screening by disc diffusion method using vancomycin (30 μg) drug. A total of 3 (2.3%) isolates of *E. faecalis* were showing intermediate resistant to vancomycin by disc diffusion method. All 3 isolates showed MIC of >4μg/ml for vancomycin. Only 2 *E. faecalis* isolates (1.5%) were positive with PCR resulted amplicon band at 402 bp for *vanC1*. None of the isolates were positive for *vanA* and *vanB* genes.

लघु सारांश

8 लघु सारांश

वर्तमान अध्ययन सूअर, का मांस और उनके पर्यावरण से एंटरोकॉसी आइसोलेट्स में वैनकोमाइसिन प्रतिरोध की पहचान करने के लिए किया गया था और एंटरोकोकी आइसोलेट्स में वैनकोमाइसिन कोडिंग जीन (वैनए, वैनबी और वैनसी1) का जीनोटाइपिक रूप से पता लगाने के बाद फेनोटाइपिक रूप से चिन्हित किया गया था। इसके अलावा, उत्तर प्रदेश के बरेली जिले और आंध्र प्रदेशक के कृष्णा जिले से एकत्र किए गए सुअर के नमूने खेंतों से एंटरोकोकी आइसोलेट्स से एंटीबायोटिक संवेदनशीलता के साथ विभिन्न महामारी विज्ञान कारकों का संबंध निर्धारित किया गया था। वर्तमान अध्ययन में 250 नमूनों से कुल 160 एंटरोकोकी आइसोलेट्स को अलग किया गया था जिसमें सुअर का मल, पर्यावरण, सूअर का मांस और मानव हाथ की सूजन शामिल थी और एंटीबायोटिक प्रतिरोध के लिए परीक्षण किया गया था। सभी 160(64%) आइसोलेट्स बीईए अगर में स्ट्रीकिंग पर ब्लैक हेलो का उत्पादन कर रहे थे और जैव रासायनिक लक्षण वर्णन द्वारा इसकी पुष्टि की जाती है और 130 आइसोलेट्स को *ई. फकियम* (एन=67) और *ई. फेकलिस* (एन=63) पीसीआर द्वारा पुष्टि की गई थी। 130 एंटरोकोकी आइसोलेट्स में से 45 को एपी के कृष्णा जिले से 85 को यूपी के बरेली जिले से अलग किया गया था। 130 एंटरोकूसी आइसोलेट्स में से 67 को जानवरों के मल से, 26 को पर्यावरण से, 20 को पोर्क से अलग किया गया था, 17 को मानव हाथ से अलग किया गया था। एंटीबायोटिक संवेदनशीलता परीक्षण से पता चला कि सभी एंटरोकॉसी आइसोलेट्स ऑक्सैसिलिन (100%) के लिए प्रतिरोधी थे, इसके बाद सबसे आम रिफैम्पिसिन (67.7%), एज़िथ्रोमाइसिन (50.8%), टेट्रासाइक्लिन (47.7%), एरिथ्रोमाइसिन (43.1%), जेंटामाइसिन (38.5%) है। सिप्रोफ्लोक्सासिन (21.5%), उच्च स्तरीय स्ट्रेप्टोमाइसिन (14.6%), पेंसिलिन (12.3%), लाइनजोलिड (8.5%), नाइट्रोफुरेंटोइन (6.2%), और टेकोप्लैनिन (3.8%)। 130 आइसोलेट्स में से 50 (38.46%) आइसोलेट्स मल्टीपल ड्रग रेसिस्टेंट टाइप के पाए गए। बायोफिल्म निर्माण के लिए परीक्षण किए गए सभी 130 एंटरोकॉसी आइसोलेट्स से पता चला है कि, 11(8.46%), 15(11.54%) और 25 (19.23%) आइसोलेट्स में क्रमशः बायोफिल्म बनाने की बजबूत, मध्यम और कमजोर क्षमता थी। एंटरोकॉसी के सभी 130 आइसोलेट्स को वैनकोमाइसिन (30 माइक्रोग्राम) दवा का उपयोग करके डिस्क प्रसार विधि द्वारा स्क्रीनिंग के अधीन किया गया था। ई. फैकलिस के कुल 3(2.3%) आइसोलेट्स डिस्क प्रसार विधि द्वारा वैनकोमाइसिन के लिए मध्यवर्ती प्रतिरोधी दिखा रहे थे। सभी 3 आइसोलेट्स ने वैनकोमाइसिन के लिए एमआईसी>4 माइक्रोग्राम/एमएल दिखाया। वैनसी1 के लिए 402 बीपी पर पीसीआर परिणामी एम्प्लिकॉन बैंडके साथ केवल 2 ई. फेकलिस आइसोलेट्स (1.5%) सकारात्मक थे। वैनए और वैनबी जीन के लिए कोई भी आइसोलेट्स सकारात्मक नहीं थे।

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Appendix

APPENDIX

I.MEDIA:

1. Nutrient agar (HiMedia-M001): -

Composition

Ingredients	Gms/Litre
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extracts	1.5
Agar-agar	15.0

28.0 gm powder was suspended in 1000 ml distilled water as per the manufacturer's (HiMedia Laboratories Pvt. Ltd., Mumbai) directions. The media was heated to dissolve the powder completely and then autoclaved at 15 lbs (121ºC) for 15 min. Once cooled aliquoted approximately 15-20 ml into each sterile 90 mm petri plate and allowed to set at room temperature.

2. Luria Bertani Broth (HiMedia-M1245):-

Composition

Ingredients	Gms/Litre
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	10.0

25.0 gm powder was suspended in 1000 ml distilled water as per the manufacturer's (HiMedia Laboratories Pvt. Ltd., Mumbai) directions. The broth was heated to dissolve the powder completely and then autoclaved at 15 lbs (121ºC) for 15 min.

3. Bile esculin azide agar (HiMedia-M1150): -

Composition

Ingredients	Gms/Litre
Casein enzymic hydrolysate	17.000
Peptic digest of animal tissue	3.000
Yeast extract	5.000
Oxgall	10.000

Sodium chloride	5.000
Sodium citrate	1.000
Esculin	1.000
Ferric ammonium citrate	0.500
Sodium azide	0.250
Agar	1.000

56.25 gm powder was suspended in 1000 ml distilled water as per the manufacturer's (HiMedia Laboratories Pvt. Ltd., Mumbai) directions and pH was adjusted to 7.2±0.2. Then autoclaved at 15 lbs (121ºC), for 15 min. Once cooled distributed in petri plates.

4. Mueller- Hinton agar (HiMedia-M173): -

Composition

Ingredients	**Gms/Litre**
Beef extract	3.0 gm
Casein hydrolysate	17.5 gm
Starch	1.5 gm
Agar-agar	17.0 gm

38.0 gm powder was suspended in 1000 ml distilled water as per the manufacturer's (HiMedia Laboratories Pvt. Ltd., Mumbai) directions and pH was adjusted to 7.4±0.2. Then autoclaved at 15 lbs (121ºC), for 15 min. Once cooled distributed in petri plates.

II. Stains and dyes

1. Gram's stain

a. Ammonium oxalate crystal violet

Solution 1: Crystal violet 2.0 gm Ethyl alchocol (95 percent) 20.0 ml

Solution 2: Ammonium oxalate 0.8 gm Distilled water 80.0 ml Solution 1 and 2 were mixed well and then filtered.

b. Lugol's (Gram's) iodine solution

Iodine	1.0 gm
Potassium iodide	2.0 gm

The ingredients were disolved and then filtered.

c. Acetone or Ethyl alchocol (decolorizer)

d. Safranin (counter stain)

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

2. 0.1% crystal violet dye

Crystal violet	100 µg
Sterile Distilled water	100 ml

I. Solutions for Agarose Gel electrophoresis

1) Stock solution – 5x TBE buffer

Working solution – 0.5 x TBE (500 ml)

TBE (5x)	50.0 ml
Distilled water	450 ml

2) Ethidium Bromide Dye – (10 mg/ml)

Working solution	5 μg/10.0 ml

3) Loading Dye – (6x)

Xylene cyanol	0.25%
Bromophenol blue	0.25%
Glycerol	30.0%

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