Characterization of MDR *Enterococcus* species of animal, human and environmental origin with special emphasis on vancomycin resistance

BY

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B.V.Sc. & A.H.

I. D. No: GVM/2017 - 33

Thesis Submitted to the

SRI VENKATESWARA VETERINARY UNIVERSITY

In partial fulfillment of the requirements

for the award of the degree of

MASTER OF VETERINARY SCIENCE

(VETERINARY PUBLIC HEALTH AND EPIDEMIOLOGY)

IN THE FACULTY OF VETERINARY SCIENCE

DEPARTMENT OF VETERINARY PUBLIC HEALTH AND EPIDEMIOLOGY

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NOVEMBER - 2019

CERTIFICATE

Mr. GOTTAPU CHAITANYA (I.D. No: GVM/2017–33) has satisfactorily prosecuted the course of research and that the thesis entitled **"CHARACTERIZATION OF MDR *Enterococcus* SPECIES OF ANIMAL, HUMAN AND ENVIRONMENTAL ORIGIN WITH SPECIAL EMPHASIS ON VANCOMYCIN RESISTANCE"** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any University.

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This is to certify that the thesis entitled **"CHARACTERIZATION OF MDR *Enterococcus* SPECIES OF ANIMAL, HUMAN AND ENVIRONMENTAL ORIGIN WITH SPECIAL EMPHASIS ON VANCOMYCIN RESISTANCE"** submitted in partial fulfillment of the requirements for the degree of **MASTER OF VETERINARY SCIENCE** for Sri Venkateswara Veterinary University, Tirupati, is a record of the bonafide research work carried out by **Mr. GOTTAPU CHAITANYA, I.D.No: GVM/2017-033** under our guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part has been fully acknowledged. All the assistance and help received during the course of investigations have been duly acknowledged by the author of the thesis.

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Acknowledgement

"Research is to see what everybody else has seen and to think what nobody else has thought"

Albert Szent-Gyorgyi

Language, though a very potent medium of expression, fails short many times, especially when feelings of gratitude and gratefulness are to be expressed. Traditional and formal words are in plenty but their 'traditional use' has turned this pleasant duty into formality. This formidable view will not project the picture of volcano of feelings that is rumbling and struggling to burst out of me through words to express my deep sense of gratitude to many known and unknown hands which pushed me forward to colour the mosaic of dissertation with the tiles of their knowledge, expertise and rational criticism, lips put elixir in my heart, learned souls put me on the right paths and enlightened me with their experience, knowledge and wisdom.

At the outset of this epistle, I take this opportunity to express my profound sense of gratitude and indebtedness to my honourable and enthusiastic guide, Chairman of Advisory Committee, ***Dr.Ch. Bindu Kiranmayi,*** *Ph.D, Assistant Professor, NTR College of Veterinary Science, Gannavaram for her unstinted support, worthy counsel, indefatigable guidance, prudent planning, innovative ideas, creative criticism, masterly suggestions, unbounded sympathy, benignity, parental encouragement, her ever willing spontaneous helping hand, whenever needed and last but not the least for being the first choice of clarifying my doubts have made her an unforgettable personality from whom I have and shall continue to imbibe essential qualities. She is not only a scientist with deep vision but also and most importantly a kind person. Her trust and scientific excitement inspired me in the most important moments of making right decisions. I feel extremely honoured for the opportunity bestowed upon me to work under her meticulous guidance.*

I owe my special thanks to ***Dr. T. Srinivasa Rao****, Associate Professor and Head, Department of Veterinary Public Health and Epidemiology, NTR College of Veterinary Science, Gannavaram for his able guidance, persistant, encouragement, positive attitude and never ending moral support under all the circumstances. I consider myself very fortunate to have worked under such a student friendly, focused and wholesome member of Advisory Committee for his valuable advice and generosity. No words could express my gratitude to*

you sir. I can't say thank you enough for your tremendous support, parental care and invaluable guidance. Without your endless encouragement and untiring interest this work would not have materialized.

I am especially indebted to ***Dr. K. Aswani Kumar, Professor, Department of Veterinary Biochemistry, N.T.R.C.V.Sc, Gannavaram*** *member of Advisory Committee for his patience, precious guidance and advice during molecular analysis. For his unflinching interest, relentless and untiring efforts, valuable advice, close supervision and simulating discussion that improved the quality of this work.*

Words fall short in this lexicon to express my gratitude to ***Dr. V.D.P. Rao, Retired Professor****. Sir, the energy you have for your profession and the true interest in the field of research is incredible and truly inspiring. Not many people could live up to your pace and consistently over the years – being an icon for professionalism.*

This dissertation will not be complete in any respect if I don't express my sincere and whole hearted sense of reverence to ***Dr. N. Subhashini,*** *Assistant Professor, Department of Veterinary Public Health and Epidemiology, N.T.R.C.V.Sc, Gannavaram for her kind cooperation, unflinching interest, valuable advice, hearty encouragement, constant supervision, helpful criticism and affection during the entire period of research work.*

Words are inadequate in the available lexicon to express my sincere thanks to ***Dr. M. Muralidhar****, Assistant Professor, Dept. of Animal genetics and breeding, for his scholastic guidance, magnanimous nature, relentless efforts, constructive counsel, generosity and keen interest at every stage of my research period in spite of his busy day to day routine.*

I owe my special thanks to ***Dr. Srinivas Prasad, Dr. Iqbal Hyder, Dr. Vasantha Seshu Kumari*** *and* ***Dr. Nikil Kumar Tej*** *for their help and support in successful completion of my research work.*

My dear colleague ***Dr. B. Swathi Vimala*** *deserve special thanks and applauses for always being there for me and to initiate scientific discussions. I would really miss a colleague like you when it comes to research. The mutual understanding and passion for research which we shared is really inexpressible.*

I am thankful to my department seniors ***Dr. K. Srinivas, Dr. Suresh, Dr. Prasastha, Dr. Chaitanya, Dr. Bhavana, Dr. Prabhu Kishore, Dr. Pratap Reddy and Dr. Soma Sekhar,*** *for creating a wonderful atmosphere in the department, making me feel this as my next home. Their care and compassion towards me kept me motivated and I will always cherish our time*

spent in little chats, long discussions and late night stays in the department as a treasured memory.

A formal statement of acknowledgement will hardly meet the ends of justice in expression of my deeply felt gratitude to my beloved juniors ***Tumma Jeswanth Reddy (bro), Puli Prasad, Nikil Alavalapati, Kavin Devasikhamani, Meghana, Brundi Stark, Jyothi Saiteja and Padi Amaranth, Bomma Manohar, PK Bhai, Sahoo Bhai, Raju Bhai, Janga Anil, Pandey, Akshat, Y.S.R, Tiwari, Daniel, Roofus, Varma, Balu, Raviraja, Harikiran, Arun, Prudvi, Sai, Nagarjuna, Sri Kiran, Vishwanath, Saikam Vishnu, Boda and Pavan*** *for selfless personal support, generous encouragement and sharing happiest moments of joy. I will always cherish the nostalgic moments we shared during sleepless nights of our peak research period.*

Friends are important part of life and without them life is incomplete, so my friends deserve special thanks and applauses for keeping me socially alive and for always being there for me. I would lke to thank ***Nanada Kishore, Chelsia, Bhargav, Ashok, Proddutur Chaitanya, Sumanth, Murali, Murali sir, Saidaiah, Nagha Bhushan, Surendra, Sivarama Krishna, Kameswari, Haritha, Suneetha, Ragjni, Sirisha, Deepthi, Hema Deepthi, Mounika, Jaya Madhuri, Yamuna, Devika, Ramya and Reshma*** *for unceasing encouragement, help, perpetual support and sharing happiest moments of joy throughout my research work as well as my stay at Gannavaram.*

Words in dictionary are not sufficient to express my sincere gratitude to my lovely seniors ***Dr. Phani kumar, Dr. Sujatha, Dr. Ramya, Dr. Ramesh, Dr. Giridhar, Dr. Vidya Sushmitha, Dr. Bharatham Prasanna, Dr. Tejaswini Reddy, Dr. Salomn Singh, Dr. Mahesh, Dr. Sowjanya, Dr. Srilekha and Dr. Roja*** *for their constant encouragement. Special thanks to* ***Dr. Siddhartha*** *for his expert tips in presentation of illustrations.*

I have been fortunate enough to have the co-operation from galaxy of friends like ***Dr. Chendu Bharath, Dr. Vineesha, Dr. Ram, Dr. Vatsala and Dr. Karthikeyan.*** *I extend my cordial thanks for their timely help, boundless love, caring and inspiration throughout my study.*

I also have invaluable assistance of my juniors ***Saikiran, Lavanya, Sudha Rani, Prakash, Gowtham, Adithya Narayan, Tejaswani, Navya, Umar, Bokka Ajay, Goli Ranga Sai Chandra, Hussain, Kavya, Divya, Laxmi Kalyani, Divya, Venkateswarlu, Prasanna, Sandeep raju, Shilpa, Shalome, Bujii, Monika, Charitha, Kanna Babu, Jaya Vani, Sravani, Sravyapriya, Lakki, Anusha, Murthy, Shruthi, Dayana, Rehman, Afreen, Bhargav,***

Madhavi, Pratyusha, Keerthana, Santosh, Raja Sambhamurthy, Sowjanya and Anjali *for their company and time to time help throughout research work for which I am indeed, beholder beyond words to them.*

My inexplicable gratitude goes to ***Vithal sir,*** *Librarian, NTR CVSc, Gannavaram for his timely help and cooperation. I am also thankful to* ***computer cell and office staff*** *for their helping hands.*

Instant availability without continuous presence is probably the best role a parent can play. Behind my success, there is my family architecture base of ideals, brick of emotion and roof of rituals. I deem it my immense pleasure to put on record my deep sense of gratitude towards my parents ***Gottapu Krishna Murthy Naidu and Leela Rani*** *for their love, affection, blessings, goodwill, encouragement, inspiration and sacrifice. I cannot weigh my feeling with my words and something that is inexpressible in my heart for my sister* ***Hima Bindhu*** *and my brothers* ***Bhanau*** *and* ***Dhanu*** *for their ever most love and affection.*

It is time to surface out my genuflect love and affectionate gratitude to my beloved ***Grandpa Chintala Balakrishna Murthy****, who is my architect, guide and philosopher who cheerfully endured full cooperation to me to reach the desired goals with love, care and showers of blessings during his entire life and still he is transcendental source of motivation for me in shaping my career.*

The help rendered by ***Mr. Suresh****, JRF,* ***Mrs. Vijaya Kumari****, Lab technician and Non-teaching staff* ***Mrs. Nalini, Mr Sandeep*** *and* ***Mr. Bhaskar*** *Department of Veterinary Public Health and Epidemiology and other members of Department of Livestock Farm Complex and Department of Livestock Products Technology, N.T.R.C.V.Sc, Gannavaram is gratefully acknowledged.*

I would like to thank The Vice Chancellor, SVVU and The Associate Dean, NTR College of Veterinary Science for giving me this opportunity to spend two fruitful years in this prestigious institution with amiable environment.

Last but not the least I would like to express my gratitude to almighty god for his blessings, right from the beginning of my life up to present situation and whatever good may come.

Place:- ***Gannavaram***

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DECLARATION

I, **Mr. GOTTAPU CHAITANYA, I.D.No: GVM/2017-33,** hereby declare that the thesis entitled **"CHARACTERIZATION OF MDR *Enterococcus* SPECIES OF ANIMAL, HUMAN AND ENVIRONMENTAL ORIGIN WITH SPECIAL EMPHASIS ON VANCOMYCIN RESISTANCE''** submitted to Sri Venkateswara Veterinary University for the degree of MASTER OF VETERINARY SCIENCE is the result of original research work done by me. It is further declared that the thesis or any part thereof has not been published earlier in any manner.

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Title of the thesis : **Characterization of MDR *Enterococcus* species of animal, human and environmental origin with special emphasis on vancomycin resistance**

Degree to which it is submitted : **Master of Veterinary Science**

Faculty : **Faculty of Veterinary Science**

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Year of submission : **2019**

ABSTRACT

The present study was undertaken to characterize *Enterococcus* species of animal, human and environmental origin based on cultural isolation. It was also aimed to determine the major antibiotic resistant genotypes (vancomycin resistance, high level aminoglycoside resistance and β- lactamase resistance), phenotypic virulence factors and virulence genes of *Enterococcus* species of worldwide public health importance. A total of 780 samples were collected including animals, foods of animal origin, water, human faecal, human diarrhoeic and human urine samples and were examined for presence of *Enterococcus* spp. i.e. *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus*. Overall prevalence of genus *Enterococcus* was found to be 86.79% and the prevalence of the *Enterococcus* spp. in various samples ranging from 100% each in sheep rectal swabs, pig rectal swabs and human diarrhoeic samples to 55.00% in uterine discharges of cattle.

Enterococci are opportunistic pathogens and cause occasional infections. Virulence gene and phenotypic virulence factors are major indicators to pathogenicity

of these microorganisms. In present study, presence of phenotypic virulence factors among 608 *Enterococcus* isolates i.e. hemolysis of sheep RBC, slime layer formation, lipase activity, caseinase activity, biofilm formation, gelatinase, DNase activity and HA test were detected in 312 (51.31%), 243 (39.96%), 47 (7.73%), 121 (19.90%), 236 (38.81%), 141 (23.19%), 37 (6.08%) and 87 (14.30%) of *Enterococcus* isolates, respectively. Out of seven virulence markers investigated in *Enterococcus* isolates, *gelE* was predominant in 181 isolates (29.76%) followed by 180 *asa* (29.60% each), 131 *hyl* (21.54%), 117 *ace* (19.24%), 101 *efaA* (16.61%) and 68 *cyl* (11.18%).

Antibiogram profiling of 608 isolates revealed a major fraction of the *Enterococcus* isolates to be resistant to polymixin-B (95.55%) followed by ceftazidime (93.25%), erythromycin (77.63%) and streptomycin (44.07%). A total of 179 (29.44%) isolates were positive for HLAR genes and *aac(6´)Ie-aph(2˝)Ia* was the only gene detected in all isolates and 127 (20.88%) isolates were positive for *blaZ* gene. The *blaZ* gene was predominantly detected in *E. faecium* (34.63%), followed by *E. faecalis* (14.38%), *E. gallinarum* (16.50%) and *E. casseliflavus* (16.66%). A total of 608 *Enterococcus* isolates studied, 125 *Enterococcus* isolates were identified as VRE genotypically. The genes *VanB*, *VanC1* and *VanC2* were detected in 14 (11.20%), 69 (55.20%) and 42 (36.60%) *Enterococcus* isolates, respectively. None of isolates showed *VanA* gene.

A greater degree of heterogeneity was observed among 124 VRE isolates (one *E. gallinarum* isolate did not yield any bands for both ERIC-PCR and REP- PCR) of different species from different sources as revealed by presence of 122 genotypes and 123 genotypes by ERIC and REP-PCR analysis, respectively. Nineteen different *E. faecalis*, 15 *E. faecium*, 57 *E. gallinarum* and 31 *E. casseliflavus* subtypes were differentiated by ERIC-PCR, whereas 21 different *E. faecalis*, 15 *E. faecium*, 56 *E. gallinarum* and 31 *E. casseliflavus* subtypes by REP- PCR. Genotyping of VR

Enterococcus species by ERIC- PCR and REP- PCR was found to be highly significant since discriminatory power >0.9 are considered highly significant (0.9997 for ERIC-PCR and 0.9999 for REP-PCR. Cluster analysis also revealed a great degree of homogeneity among some VRE isolates recovered from different sources and implied at the chance of cross-contamination of foods of animal origin.

LIST OF SYMBOLS AND ABBREVIATIONS

β	- Beta
µg	- microgram
16S rRNA	- RNA sequence of small subunit of bacterial ribosome
ace	- Collagen- binding adhesion
AFLP	- Amplified fragment length polymorphism
asa	- Aggregation substance
ATCC	- American Type Culture Collection
bla	- Beta-lactamase coding gene
bp	- Base pairs
CDC	- Centers for Disease Control and Prevention
CFU	- Colony Forming Unit
CLSI	- Clinical and Laboratory Standards Institute
CNS	- Central nervous system
CSIR	- Council of Scientific and Industrial Research
Cyl	- Cytolysin
DNA	- Deoxyribo Nucleic Acid
dNTP	- Deoxyribo nucleotide triphosphate
EDTA	- Ethylene Diamine Tetra Acetic Acid
ERIC-PCR	- Enterobacterial repetitive intergenic Consensus-PCR
Esp	- Enterococcal surface protein
gelE	- Gelatinase
GRE	- Glycopeptide Resistant Enterococci
h	- Hour
HLAR	- High Level Aminoglycoside Resistance

HLGR - High Level Gentamicin Resistant

HLSR - High Level Streptomycin Resistant

hyl - Hyaluronidase

ICMSF - International Commission on Microbiological Specifications for Foods

IE - Infectious Endocarditis

IVRI - Indian Veterinary Research Institute

KAA - Kanamycin Aesculin Azide Agar

Kbp - Kilo base pairs

KFA - KF- streptococcal Agar

LFC - Livestock Farm Complex

MDR - Multi drug resistant

MHA - Muller Hinton Agar

MIC - Minimun Inhibitory Concentration

min - Minute

ml - milli liter

MLST - Multilocus sequence typing

m-PCR - Multiplex Polymerase Chain Reaction

MRSA - Methicillin-resistant *Staphylococcus aureus*

MTCC - Microbial type culture collection and gene bank

ng - Nanogram

PBPs - Penicillin binding proteins

PBS - Phosphate Buffered Saline

PCR - Polymerase Chain Reaction

PFGE - Pulsed Field Gel Electrophoresis

pmol/µl - Pico moles/ Microliter

Rep-PCR	- repetitive sequence-based PCR
RFLP-PCR	- Restriction Fragment Length Polymorphism-PCR
STP	- Sewage treatment plant
UK	- United Kingdom
UTI	- Urinary Tract Infections
VR	- Vancomycin-resistant
VRE	- Vancomycin resistant enterococci
VRSA	- Vancomycin-resistant *S. aureus* (VRSA)
VS	- Vancomycin Sensitive
WHO	- World Health Organization
XDR	- Extreme Drug Resistant

CHAPTER - I

INTRODUCTION

Microorganisms have a dynamic relationship with the biosphere after continually adapting to inconstant environmental conditions, thus generating an enormous amount of genetic diversity. Bacteria that inhabit animal niches can colonize and proliferate within a host and establish a similarly vigorous association. 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).

The microorganisms belonging to the genus *Enterococcus* are frequently encountered in food as contaminants due to their ubiquitous nature and ability to survive adverse environmental conditions. Enterococci, is a complex group of eubacteria that are diverse and possess a significant relationship with the humans. Enterococci are the part of normal gut flora of animal and humans. In addition, these bacteria are found in the soil, on plants, surface water and other environments exposed to human or animal faeces (Kuhn *et al*., 2003 and Fisher and Philips, 2009). Traditionally, these have been considered as good indicator organisms to predict unhygienic conditions of production and processing of foods. Some strains of economic importance are being used in the food industry (Gilmore *et al*., 2002). There are 28 species of enterococci proposed with appropriate genetic evidences (Carvalho *et al.*, 2004b). Only a limited number of enterococcal species are of importance for the ecology of the food microflora, including *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus* (Klein, 2003).

Enterococci are generally considered as harmless commensals. However, several recent reports have documented the pathogenic potential of enterococci capable of causing a variety of health problems such as urinary tract infection, bacteremia, intra-

abdominal infections and endocarditis (Moellering, 1992 and Huycke *et al*., 1998). *Enterococcus* species with the highest virulence are clinical isolates. Till date, no single virulence factor has been demonstrated to be essential for enterococcal infections. Many factors may determine the virulence of *Enterococcus* species like, their ability to colonize the gastrointestinal tract which is the normal habitat and ability to adhere to a range of extracellular matrix proteins. Several studies have shown that virulence factors namely *asa* (aggregation substance), *esp* (enterococcal surface protein) and *ace* (collagen- binding adhesin) are associated with colonization of *Enterococcus*. Secreted virulence factors like gelatinase, DNase, haemagglutinin, lipase and haemolysin are the most frequently mentioned virulence determinants in the pathogenicity of human infection and in animal models (Jett *et al*., 1994 and Coque *et al*., 1995).

Multiple antimicrobial drug resistance among the enterococci further compounds the problem of enterococcus infection and presents great therapeutic challenge (Moellering, 1992). Indiscriminate use of antibiotics and growth promoters in animals may be one reason for the emergence 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 and Pavia *et al*., 2000) and there is possibility that these MDR enterococci are transmitted to man via the food chain (Bates, 1997).

Enterococci have been known for over a century for their potency to cause infections in humans (MacCallum and Hastings, 1899 and 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 the drug of choice for susceptible enterococcal infections. Emergence of resistance to multiple antibiotics and its ability 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).

In 1980, for the first time high level gentamicin resistant (HLGR) enterococci was reported from hospital settings in Connecticut (Patterson and Zervos, 1990). Subsequently, ampicillin resistant *E. faecium* started to emerge (Galloway-Pena *et al*., 2009) and in 1986 high-level VRE were discovered (Leclercq *et al.*, 1988). But in the last two decades its position as a major opportunistic pathogen contributing to an increased level of nosocomial infections is found to be escalating. Enterococci are reported to be the most frequently isolated bacteria from hospital associated infections in the US and Europe (Hidron *et al*., 2008).

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 penicilins (Marothi *et al*., 2005). The prime reason for this appears to be the ability of the organism to acquire resistance determinants from related strains and spontaneous mutations within the bacterium (Patterson and Zervos, 1990). HLGR has been a cause of concern in many hospital associated infections. There has been a tenfold increase in the prevalence of HLGR enterococci during 2003 – 2008 (Rosvoll, 2012).

Recent studies have even revealed remarkable resistance of enterococci to the glycopeptide antimicrobials like vancomycin and teicoplanin in the clinical samples of human origin. The presence of VRE has also been recorded in foods (Pavia *et al*., 2000 and Gambarotto *et al*., 2001). The ability of enterococci to transfer vancomycin resistance to other common pathogens like streptococci, staphylococci and others, may pose further serious adverse public health consequences (Franke and Clewell, 1981).

The pathogenic potential of clinical isolates of enterococci through different models has been demonstrated (Miyazaki *et al*., 1993) and the presence of a number of virulence factors has also been reported (Dupont *et al*., 1998). However, such information on the isolates of food origin is not readily available in India. Though the role of antibiotic resistant strains of enterococci has been demonstrated in clinical cases in human beings (Khanal *et al*., 1998 and Purva *et al*., 1999), much attention has not been paid.

In this context, the present study was proposed with following objectives –

1. To isolate and characterize different *Enterococcus* species from various meat, animal faeces, human diarrhoeic and environmental samples.
2. To characterize virulence factors of enterococci isolates by phenotypic and genotypic methods.
3. To study antimicrobial resistance profile and vancomycin resistance in enterococci isolates by phenotypic and genotypic methods.
4. To assess genetic diversity of VRE by using REP-PCR and ERIC-PCR.

CHAPTER - II

REVIEW OF LITERATURE

2.1 THE GENUS *ENTEROCOCCUS*

Enterococcus is a large genus of lactic acid bacteria of the phylum Firmicutes. Enterococci are Gram-positive, ovoid, non-motile and non-sporing bacteria occurring either singly, in pairs or as short chains and are difficult to distinguish from Streptococci on physical characteristics alone. Two species of enterococci are common commensal organisms in the intestines of humans: *E. faecalis* (90–95%) and *E. faecium* (5–10%). Rare clusters of infections occur with other species, including *E. casseliflavus*, *E. gallinarum* and *E. raffinosus* (Gilmore *et al.*, 2002).

Enterococci are facultative anaerobic chemoorganotrophs with complex nutritional requirements and a fermentative metabolism resulting in lactic acid as the major product of glucose fermentation. The optimum temperature for growth is 37^0C. Majority of *Enterococcus* species are able to grow at 10^0C and 45^0C, survive heating at 60°C for 30min and can grow in 6.5% NaCl at pH 9.6. They are also tolerant to 40% bile. Most species are characterized by the possession of the Lancefield group D antigen. Enterococci are distributed widely in nature. Their habitat is diverse and they are commonly found in the gastrointestinal tract of man, other mammals, birds, reptiles, insects, plants, soil and water (Deibel and Hatman, 1984).

2.2 DISCOVERY OF ENTEROCOCCI – A ROAD MAP

The history of Enterococci dates back to a century when Thiercelin (1899) used the term "*enterocoque*" in a French publication to describe bacteria seen in pairs and short chains in human feces. Andrewes and Horder (1906) first coined the name *Streptococcus faecalis*, for an isolate recovered from blood of a patient with

endocarditis and considering that it was “so characteristic that of human intestinal origin” (Biswas, 2015).

Orla-Jensen (1919) described a second organism of this group, *Streptococcus faecium* that differed from the fermentation patterns of *S. faecalis.* A third species *S. durans* was proposed by Sherman and Wing (1935), which was similar to *S. faecium* but has less fermentation activity. In 1937 Sherman emphasized that the term *Enterococcus* had been used to mean different things ranging from the broad definition of any faecal *Streptococcus* to a restricted definition of organisms that appeared to be identical to *S. faecalis*. He proposed a classification scheme, which seperated streptococci into four divisions: pyogenic, viridians, lactic and *Enterococcus*. Sherman’s classification scheme also correlated with the serological scheme originated by Lancefield in the early 1930s (Lancefield, 1933), where the enterococci reacted with group D antisera.

Kalina (1970) proposed a separate genus “*Enterococcus*” for the enterococcal species based on cellular arrangement and phenotypic characteristics. 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 members 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. avium, E. casseliflavus, E. durans, E. malodoratus* and *E. gallinarum* for those species. As of August 2004, there are 28 species of enterococci proposed with appropriate genetic evidences (Carvalho *et al.*, 2004b)

2.3 HABITAT AND ENVIORNMENTAL SIGNIFICANCE OF ENTEROCOCCI

Enterococci are considered as important members of the intestinal microflora of mammals, reptiles, birds, fish and insects as well as in plant environments. Human and animal wastes are disposed into the environment through sewage or nonsewage systems. Animal wastes are normally used untreated as fertilizers on fields. So, presence of *Enterococcus* spp. in water and food indicates faecal contamination. In water, the species considered as faecal contaminants are mainly *E. faecium* and *E. faecalis* 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). Thus enterococci may survive some types of food processing and have been implicated in food spoilage of processed cooked meat. More specifically, *E. avium, E. durans, E. faecalis* and *E. faecium* are frequently isolated from cheese products whereas *E. avium*, *E. casseliflavus*, *E. durans*, *E.faecalis*, *E. faecium*, *E. gallinarum* and *E. hirae* have been described components of the microbiota of various raw meat products (Carvalho *et al*., 2004a**).**

In humans, typical concentrations of enterococci in stool are up to 108 Colony Forming Units per gram. Although the oral cavity and vaginal tract can become colonized, enterococci are recovered from these sites in fewer than 20% of cases (Murray, 1990, Kuhn *et al.,* 2003 and Mondino *et al.,* 2003).

2.4 PREVALENCE OF *ENTEROCOCCUS* SPP.

Enterococci have been detected from many domestic animals and wild animals, as well as in poultry (domestic and wild birds) and they serve as source of infection to humans (Chandra, 2003).

Few early reports have suggested the food poisoning potential of these organisms (Cantoni and Bersani, 1988).Though, there is no consensus on whether

enterococci pose threat as food poisoning organisms, there are reports of animals harboring antibiotic resistant strains of enterococci suggesting the possibility of spread of these organisms via the food chain (Van Den Bogaard *et al.,* 1997). Pavia *et al.* (2000) demonstrated widespread dissemination of glycopeptide-resistant enterococci strains in meat in Italy.

2.4.1 Prevalence of enterococci in milk and dairy products

Microorganisms gain access into milk and milk products either through contaminated water supply or during unhygienic production and handling. Enterococci are frequently encountered in dairy products including milk, cheese, ice-cream and also those which undergo considerable heat-treatment e.g., dried milk, infant foods and pasteurized milk due to their ability to survive pasteurization temperature (Chandra, 2003).

Das *et al.* (1986) reported 62 isolates of enterococci from milk and milk products including raw milk, pasteurized milk, milk cake, burfi, peda and dried skim milk. Out of 62, 28 (46.66%) were *E. faecalis*, 4 (6.45%) *E. faecalis* subsp. *liquefaciens*, 3 (4.83%) *S. faecalis* variants, 2 (3.22%) *E. faecium* variants and 12 (19.35%) *E. durans* isolates.

Wessels *et al.* (1988) reported that *E. faecium* was predominant species isolated from various dairy products mainly in yoghurt (100%) and sour milk (l00%), followed by *E. faecalis* in cream (84.6%) and butter (41.2%) and *E. durans* isolates in butter.

Citak *et al.* (2000) investigation revealed the presence of enterococci in 9.52% samples of pasteurized milk collected in Ankara (Turkey). Medina *et al.* (2001) reported that *Enterococcus* spp. constituted 48% of the lactic acid bacteria present in ewe's milk.

Chingwaru *et al.* (2003) isolated 970 *Enterococcus* isolates from 227 raw and pasteurized milk samples. These included *E. faecalis* (42.8%), *E. faecium* (28.2%),

E. gallinarum (7.9%), *E. avium* (6.2%), *E. durans* (2.5%), *E. hirae* (2.4%), *E. casseliflavus* (9.6%) and *E. mundtii* (0.4%).

Citak *et al.* (2005) reported that out of 177 enterococci isolated from 78 raw milk samples, *E. faecalis* (54.2%) were the most frequently isolated species, followed by *E. faecium* (29%), *E. durans* (6.2%), *E. hirae/dispar* (5.0%), *E. gallinarum groups* (3.0%), *E. mundtii* (2.2%) and *E. raffinosus* (0.5%).

2.4.2 Prevalence of enterococci in meat of animal origin

Consequent to their presence in the gastrointestinal tract of animals and in the environment, enterococci are the usual contaminants in meat as a result of unhygienic slaughtering, processing and handling (Chandra, 2003).

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, 3 *E. faecium*, 3 *E. gallinarum* and 1 *E. casseliflavus*.

Chandra and Garg (2006) analysed 37 samples of meat including chicken (10), chevon (11), pork (10) and cara beef (6) for presence of enterococci. The positive samples usually carried more than one species of genus enterococcus. A total of 35 enterococci isolates were isolated where *E. faecalis* (73%) was the most prevalent species, followed by *E. gallinarum* (45.9%) and *E. raffinosus* (37.8%). Other species included *E. faecium*, *E. durans*, *E. hirae*, *E. mundtii*, *E. solitarius*, *E. pseudoavium*, *E. dispar*, *E. cecoruum* and *E. avium*.

2.4.2.1 Prevalence of enterococci in chicken meat

Oliveira *et al*. (1999) reported prevalence of *E. faecalis*, *E. faecium* and *E. avium* to be 77%, 6.7% and 16.7% in chicken hamburger samples sold in Rio de Janeiro, respectively.

Chingwaru *et al.* (2003) isolated 228 *Enterococcus* isolates from 58 chicken samples sold in Gaborone and Botswana. These included 107 (46.9%) *E. faecalis*, 75

(32.9%) *E. faecium*, 16 (7.0%) *E. gallinarum*, 12 (5.3%) *E. avium* and 18 (7.9%) *E. casseliflavus*.

2.4.2.2 Prevalence of enterococci in pork

Knudtson and Hartman (1993) examined pork carcasses during different stages in the slaughter process as well as on pork products (3 hog slaughtering plants in Lowa state) and reported that *E. faecalis* was more prevalent than *E. faecium*. 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).

Pavia *et al.* (2000) reported isolation of 8 (33.3%) enterococci isolates from 24 pork samples collected from retail outlets of Catanzaro, Italy.

2.4.2.3 Prevalence of enterococci in beef

Klein *et al.* (1998) isolated 209 enterococci strains from minced beef (275) in Berlin, Germany which included 182 *E. faecalis* isolates, 8 *E. faecium* and 3 each of *E. gallinarum*, *E. durans* and *E. hirae*.

Ingham and Schmidt (2000) evaluated the sanitary condition of beef carcasses obtained from Madison slaughter house plant over 6 months and they concluded that 31.9% of beef carcasses contained *Enterococcus* species.

2.4.3 Prevalence of *Enterococcus* spp. in water and sewage samples

Due to their ubiquity in human feces and persistence in the environment, enterococci have been adopted as indicators of human fecal pollution in water. More recently, their densities on human hands have been used as indicators of hand hygiene. The use of enterococci as indicators of human fecal pollution or contamination can be problematic, however, because enterococci are also found in animal feces (Harwood *et al.*, 2000 and Layton *et al.*, 2010) in soils (Byappanahalli and Fujioka, 2004 and Goto and Yan, 2011) and on plants (Byappanahalli *et al.*, 2003).

Kimiran-Erdem *et al*. (2007) collected seawater samples from coastal areas of Istanbul and isolated around 100 enterococci species of which 96 were *E. faecalis*, three *E. gallinarum* and one *E. solitarius*.

Jahangiri *et al*. (2010) isolated 156 *Enterococcus* spp. from sewage treatment plants (STP) of which, 58 were Vancomycin-Sensitive (VSE) *E. faecium* and 98 VR *E. faecium*.

Alipour *et al*. (2014) reported that 70 enterococci isolates were recoverd from the Babolrud River and coastal waters of Babolsar. They reported that *E. faecalis* (68.6%) and *E. faecium* (20%) were the most prevalent species and were showing resistance to chloramphenicol, ciprofloxacin and tetracyclin.

Enayati *et al*. (2015) examined 15 different water samples collected from 9 private wells and 6 rivers located east of Tehran and reported presence of 315 enterococci isolates. Out of 315 isolates, *E. faecium* (118) was the predominant species followed by *E. galinarum* (110), *E. hirea* (37), *E. casselifelavus* (32) and *E. mundeti* (18).

Khan *et al.* (2005) reported occurrence of 30 MDR *Enterococcus* spp., 2 Strains from mastitis cow milk, 9 from chicken litter and 19 from turkey litter. Out of 30, 25 were identified as *E. gallinarum* and 5 as *E. faecalis*.

2.5 PUBLIC HEALTH SIGNIFICANCE OF ENTEROCOCCI

There are around 28 identified species of enterococci (Carvalho *et al*., 2004b). Of these, *E. faecalis* and *E. faecium* are the most important causes of enterococcal infections in humans. The pathogenic potential of enterococci was first recognized by MacCallum and Hastings (1899) who isolated an organism from a case of acute endocarditis and designated it as *Micrococcus zymogenes*. Now, about a century later, enterococci have emerged as significant pathogens capable of causing a diverse variety of community-acquired and hospital-acquired infections in human beings. During the

last two decades, *Enterococcus* species have particularly acquired a prominent position as emerging pathogens. The National Nosocomial Infections Surveillance Report declared enterococci to be ranking second only to *Escherichia coli* (NNIS Report, 2004). Enterococci have been listed as the third most common cause of nosocomial bacteremia, accounting for 12.8% of all isolates (NNIS Report, 1997).

UK National Prevalence Survey has also indicated increase in enterococcal infections in the UK (Morrison *et al.,* 1997). Among different enterococci species, *E. faecalis* constitutes 85 to 90% of the clinical isolates in most of the hospital acquired infections while *E. faecium* accounts for 5 to 10%. Reports of infections due to *E. durans, E. avium, E. gallinarum* and *E. casseliflavus* are also increasing but these are encountered far less than above two (Moellering, 1992).

Enterococci are responsible for causing a diverse range of infections. Among these, urinary tract infections (UTI) are the most common ones and majority of these are 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). The enterococci are also known to cause occasional neonatal, CNS and respiratory tract infections (Murray, 1990). Enterococci constitute third leading cause of infective endocarditis (IE) and account for 5-20% cases of native valve IE and 6-7% of prosthetic valve IE (Megran, 1992).

In India, resistance of clinical enterococcal isolates to commonly used antimicrobial drugs particularly from wound infections has been frequently observed (Jesudason *et al.,* 1998). In India, Khanal *et al.* (1998) recorded involvement of HLGR enterococci in IE. Enterococcal infections occur both by endogenous and exogenous route (Moellering, 1992 and Morrison *et al.,* 1997) but there are increased recent reports of exogenous spread of MDR enterococci in hospital environment (Moellering, 1992).

Bertrand *et al*. (2000) reported isolation of *E. faecalis* from urinary tract specimens (64%), superficial swabs (12.5%), surgical wounds (3%), blood (2%), and other specimens (18.5%) from 225 patients in Eastern France.

Baran *et al*. (2001) showed the prevalence of recurrent VRE bacteremia. Of 36 inpatients who had episodes of bacteremia, 3 (8.3%) had recurrent episodes. In Slovakia, they reported death of 40 (39.9%) patients of enterococcal bacteremia was reported due to inappropriate therapy.

Bouza *et al.* (2001) isolated 607 microorganisms from 522 patients with nosocomial UTI (urinary tract infection) and reported *Enterococcus* to be the second most important organism (15.8%) after *E. coli* (35.6%) in Madrid, Spain.

Desai *et al.* (2001) recorded the prevalence of enterococci in burn wounds (29.51%), ascitic fluids (7.1%), Foley's catheters (48.12%), urine (8.92%), non-surgical wounds (17.77%), surgical wounds (21%), umbilical stumps (22.22%) and abdominal drain fluid (0.1%).

Miskeen and Deodhar (2002) studied the incidence of *Enterococcus* spp. in UTIs. Enterococci were isolated in pure cultures from 147 specimens (7.38%) out of 4030 urine specimens. *E. faecalis* was the most predominant species (128, 87%), followed by *E.faecium* (16, 10.88%) and *E. durans* (3, 2.04%).

2.6 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 presence in large numbers in the incriminated foods and the absence of other pathogens such as *S. aureus* or *Salmonella* spp (Riemann and Bryan, 1979). On the other hand, it is felt that enterococci can cause food intoxication through the production of biogenic amines, but both of these observations are yet to be confirmed (Giraffa, 2002).

Water contaminated with enterococci with virulence properties and antibiotic resistance can be a potential source of risk for the consumers (Peter *et al*., 2012). Sources of enterococci in recreational waters include sewage, agricultural and urban runoff, stormwater, direct input by animals via defecation, bather shedding, boats, plant debris, polluted groundwater, soils, sediments and sands. In developed countries, sewage is typically well-treated prior to discharge through an outfall that is usually located far 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*., 2008).

2.6.1 Nosocomial trasmisson

Common modes of nosocomial spread are either endogenous, exogenous or environmental surfaces. Endogenous infections are acquired from patient's own bacterial flora, exogenous from other patients or healthcare workers and environmental surfaces such as contaminated bed rails, sinks or door knobs. The major factors in the spread of infection are: number of organisms shed from the source, their ability to survive after leaving the source, their virulence and the means of reaching a susceptible site on the same or other person in sufficient numbers to cause infection (Ayliffe *et al*., 1982).

2.7 ISOLATION AND PHENOTYPIC IDENTIFICATION OF *ENTEROCOCCUS* SPP.

Due to their significance in food, feed, environmental and clinical samples, the detection and enumeration of enterococci has become an important issue not only in daily routine but also in current research activities. Several media and protocols have been developed for diverse purposes, but there is no single method, which universally meets all requirements. 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).

2.7.1 Media used for the detection of enterococci from different samples

Basically, the choice of a particular medium depends on whether enterococci are to be counted in total and whether the habitat is highly contaminated or not (Reuter, 1992 and Reuter, 1995). Garg and Mital (1991) analyzed several media and concluded that there is no ideal media available for the isolation of enterococci from foods, because most media display drawbacks in terms of selectivity and recovery. They also reported that Kenner Fecal agar (KF agar) is a suitable compromise and frequently used for the enumeration of enterococci in non-dairy foods, whereas citrate azide agar is recom- mended for dairy products.

Media for the examination of enterococci are usually incubated at 35 – 37^{0}C. However, when examining enterococci in dairy products, a higher incubation temperature (45^{0}C) is necessary to suppress the growth of the background microflora (Deibel and Hartman, 1984).

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. Barton and Doern (1995) compared two selective media (Bile Esculin agar and Columbia Colistin Nalidixic Acid Blood agar) for the detection of VRE from the gastrointestinal tract and concluded that both are equally effective.

2.7.2 Media used for the investigation of enterococci in water

Membrane filter Slanetz and Bartley (SB) agar and Azide Dextrose (AD) broth are commonly used for MPN (Most Probable Number) techniques and substrates containing bile and aesculin (Bile Esculin agar and Esculin Iron agar) for confirmatory tests (Slanetz and Bartley, 1957). Those media incubated at 44.5 ^{0}C were more selective, but lower were obtained than at 37 ^{0}C (Dutka and Kwan, 1978).

2.7.3 KF streptococcal (KF) agar

Since *E. faecalis* and *E. faecium* play a dominant role in food microbiology, KF streptococcal agar was especially designed for this purpose (Kenner *et al.,* 1961) and are usually incubated for 48h at 37^0C. For dairy products, KF agar incubated for 2 days at 44^0C has to be used (Centeno *et al.,* 1996 and Medina *et al.,* 2001).

KF-streptococcus agar contains sodium azide as the main selective agent and TTC for (2,3,5-Triphenyl tetrazolium chloride) differential purposes. The medium is relatively rich in maltose (2.0% w/v) and contains a small amount of lactose (0.1% w/v). Many but not all enterococci and streptococci are able to ferment these sugars. Furthermore, the intensity of TTC reduction varies among the species. *E. faecalis* reduces TTC imparting a deep red colour to the colony, while other enterococci and streptococci appear bright pink in colour. However, some strains of *Pediococcus*, *Lactobacillus* and *Aerococcus* may grow and also produce bright pink colonies (Hartman *et al.,* 1992).

2.7.4 Kanamycin Aesculin Azide (KAA) agar

KAA is a commercially available medium, which is used for the isolation and enumeration of enterococci from foods, water and other specimens (Mossel *et al.,* 1978). Many companies and organisations have approved the KF agar to be used for the quantitative enumeration of enterococci in water and non-dairy foods and KAA agar for dairy products. It contains sodium azide and kanamycin as selective agents. Targeted enterococci hydrolyse aesculin, forming black haloes around the colonies. Usually, the medium is incubated for 24h at 37^0 C. However, increased incubation temperature 42^0C for 18h may improve the selectivity but does not inhibit the growth of aesculin-positive lactobacilli. Increasing the concentration of sodium azide may circumvent this problem but the recovery rate of enterococci will be reduced (Reuter, 1995).

2.8 MOLECULAR METHODS FOR IDENTIFICATION OF *ENTEROCOCCUS* SPECIES

Bacterial cultivation in *invitro* and followed by biochemical characterization (carbohydrate fermentation and enzyme pattern) can be regarded as a conventional way of microbial differentiation. According to recent advancements, alternative procedures have been used to simplify and to speedup these methods that are alternatively termed as "Molecular Phenotyping" since the procedures used, help in identifying and exploiting the phenotypic characteristics of the organisms, unlike genotypic methods which uses the genetic content (DNA) of the organism. The different molecular methods used for identification of *Enterococcus* species are listed in Table-1.

TABLE-1 Molecular methods applied for the identification of *Enterococcus* species (Facklam *et al*., 2002)

METHODS	REFERENCES
Whole cell protein analysis	Niemi *et al.,* 1993, Merquior *et al.,* 1994 Devriese *et al.,* 1995, Teixeira *et al.,* 1997 and Teixeira *et al.,* 2001
Vibrational spectroscopic analysis RAPD analysis	Descheemaeker *et al.,* 1997 and Quednau *et al.,* 1998 and Kirschner *et al.,* 2001
Sequencing analysis of 16S rRNA Genes	Williams *et al.,* 1991 and Patel *et al.,* 1998
Fragment length polymorphism analysis of amplified 16S rDNA	Ulrich and Muller, 1998
BR-PCR of the 16S rDNA	Monstein *et al.,* 1998
Sequencing of the domain V of 23S rRNA gene	Tsiodras *et al.,* 2000
Amplification of the rRNA intergenic spacers	Naimi *et al.,* 1997
Amplification of the Trna intergeneric spacer	Baele *et al.,* 2001
Sequencing of the *ddl* genes	Dutka-Malen *et al.,* 1992, Evers *et al.,* 1996 and Ozawa *et al.,* 2000

Amplification of *ddl* and *Van* genes	Dutka-Malen *et al.*,1995, Satake *et al.*, 1997, Kariyama *et al.*, 2000 and Angeletti *et al.*, 2001
Sequencing of the *sodA* gene	Poyart *et al.*, 2000
Sequencing and probing of the *cpn*60 gene	Goh *et al.*, 2000
Amplification and probing of the *efaA* gene	Coque and Murray, 1995, Singh *et al.*, 1998 and Duh *et al.*, 2001
Amplification and probing of the *ace* gene	Duh *et al.*, 2001
Amplification and probing of the *tuf* gene	Ke *et al.*, 1999
Amplification and probing of the *pEM1224* gene	Cheng *et al.*, 1997

2.9 PUTATIVE VIRULENCE DETERMINANTS AND VIRULOTYPING

Bacterial virulence determinants are predominantly encoded by mobile genetic elements such as phages, plasmids, insertion elements or transposons and a large number of such determinants are located within pathogenicity islands.

2.9.1 Putative virulence determinants

2.9.1.1 Cytolytic Toxin (*cylA* and *cylB*):

Commonly called hemolysin, is a post-translanslationally modified protein toxin that occurs in upto 60% of *Enterococcus* isolates. Among the target cells of cytolysin are the erythrocytes, polymorphonuclear neutrophils, macrophages and a broad range of Gram positive organisms (Johnson, 1994 and Hardie *et al.*, 2010). Enterococci cytolysin is bicomponent in nature i.e. it has two operationally defined components: L (for lysis) and A (for activator). Cytolysin producing enterococci cells resist self lysis (Segarra *et al.*, 1991).

2.9.1.2 Aggregation Substance (*asa*)

Aggregation substance is a surface bound protein which mediates the efficient contact between donor and recipient bacterium and facilitates plasmid exchange during the process of conjugation (Johnson, 1994).

2.9.1.3 Gelatinase (*gelE*)

The role of gelatinase is thought to be in producing nutrients by degrading host tissue, although they have some function in biofilm formation. Gelatinase is an extracellular zinc containing metalloproteinase which can hydrolyse gelatin, collagen, fibrinogen, casein, hemoglobin, insulin and some other bioactive peptides (Makinen *et al.,* 1989, Gilmore *et al.,* 2002 and Vergis *et al.,* 2002).

2.9.1.4 Enterococcal Surface Protein (*Esp*)

esp is associated with promotion of primary attachment and biofilm formation of enterococci on abiotic surfaces. *esp* could be responsible for hydrophobicity, hence biofilm formation and adherence to abiotic surfaces is increased. *esp* has also been shown to adhere to urinary bladder epithelial cells showing tissue specificity (Marton *et al.,* 1993; Bab *et al.,* 1997; Nallapareddy *et al.,* 2000 and Upadhayaya *et al.,* 2009).

2.9.1.5 Hyaluronidase (*hyl*):

Hyaluronidase acts on hyaluronic acid and is mainly a degradative enzyme associated with tissue damage. Hyaluronidase depolymerizes the mucopolysaccharide moiety of connective tissue and so increases bacterial invasiveness. It facilitates the spread of bacteria as well as toxins through host tissue. It paves the way for the deleterious effect of other bacterial toxins, thus increasing the magnitude of damage (Upadhayaya *et al.,* 2009).

2.9.1.6 Endocarditis Antigen (*efaA*):

Endocarditis antigen functions as an adhesin in endocarditis. Endocarditis caused by enterococci is the most therapeutically challenging. The severity of this

infection may be increased with acquisition of high level and multidrug resistance (Marton *et al.*, 1993, Bab *et al.*, 1997, Nallapareddy *et al.*, 2000 and Upadhayaya *et al.*, 2009).

2.9.1.7 Biofilm formation by enterococci

Biofilm is a population of cells attached irreversibly on various biotic and abiotic surfaces and encased in a hydrated matrix of exopolymeric substances, proteins, polysaccharides and nucleic acids. Enterococci in biofilm are more highly resistant to antibiotics than planktonically growing enterococci. *Enterococcus* has the capacity to bind to various medical devices such as ureteral stents, intravascular catheters, biliary stents, ocular lens materials like silicone and acrylic (Stephenson and Hoch, 2002). Biofilm formation encoding genes in enterococci are *agg, efaA, ace, bop, epbA, epbB, epbC, pil, srt, fsrA, fsrB, fsrC, gelE, sprE, cpd, cob* and *ccf* (Hashem *et al*., 2017).

Khan *et al.* (2005) reported 30 MDR *Enterococcus* spp. strains, two from mastitis milk, nine from chicken litter and 19 from turkey litter, out of which the genes *cylA* and *cylB* were detected only in one clinical *E. gallinarum* isolate and none of the virulence factors were found in milk or poultry isolates.

Jahangiri *et al*. (2010) screened the *Enterococcus* isolates from sewage for the presence of virulence genes, *asa1*, *cyl*, *esp*, *gelE* and *hyl* by PCR. Results showed the presence of *hyl* (3.2%) and *esp* (41%) genes in sewage isolates. Furthermore, *cyl, gelE* and *asa1* were not found in STP isolates at Pasteur Institute of Iran.

Enayati *et al*. (2015) examined 315 *Enterococcus* isolates for virulence factors (*esp, acm, gelE, asa1, cylA* and *hyl* genes) by using m-PCR. Virulence determinants were found in 84.7%, 33.9%, 16.1% and 2.5% of isolates for *acm, asa1, esp* and *cylA* genes, respectively. None of the isolates carried *hyl* and *gelE* gene.

Biswas *et al*. (2014) examined the fecal isolates for presence of *gel E, esp* and *asa1* and they were found significantly higher in VRE as compared to VSE. Correlation

between the presence of virulence genes and their expression as detected by phenotypic tests showed that biofilm production was seen in 61.1% (22/36) of clinical VRE, the corresponding genes i.e., *asa1* and *esp* were detected in 30.5% (11/36) and 27.8% (10/36) of strains, respectively.

2.9.2 Virulotyping

Virulotyping, a novel concept that has surfaced recently is a special type of genotyping where the alleles of interest are virulence genes of bacterial populations. It is often considered synonymous with pathotyping. Virulotyping possesses the discriminatory power to differentiate strains within species as virulent and avirulent and is considered advantageous over other genotyping methods in order to study the epidemiology of food borne diseases and their outbreaks. Virulotyping has been attempted in various food borne pathogens such as *Salmonella*, *E. coli*, *Listeria monocytogenes*, *Shigella* spp. and *Campylobacter* spp (Timothy *et al.,* 2008).

2.10. ANTIBIOTIC RESISTANCE PROFILING OF *ENTEROCOCCUS* SPP.

The use of antibiotics 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 animal husbandry for three reasons: prevention or prophylaxis of disease encountered by bacterial infection, treatment against serious infections and growth promotion (Schwarz and Chaslus-Dancla, 2001 and Marshall and Levy, 2011).

Antibiotics used in livestock are significantly important in human therapy. Certain antibiotics are given at low concentrations to enhance the growth of these animals. Antibiotics that are profoundly used in animal husbandry are tetracyclines, aminoglycosides, cephalosporins, fluoroquinolones, lincosamides, macrolides, pleuromutilins, penicillins, phenicols and sulfonamides (Schwarz and Chaslus-Dancla,

2001). Vital drugs used in humans are cheaply available and are used in animal rearing in several countries. However, due to a rise in resistance against several antibiotics used in human therapy, they were banned in 1998 and 1999 (Schwarz and Dancla, 2001 and Castanon, 2007). Use of antibiotics for non-therapeutic purposes can be risky to human health. Also, structurally similar antibiotics aim a common target; it may be the probable reason that confers cross resistance (Marshall and Levy, 2011).

Enterococci are less virulent organisms but despite this, enterococcal infections pose serious therapeutic challenge to clinicians due to their intrinsic and acquired resistance to antimicrobial agents. Enterococci show intrinsic resistance to cephalosporins and reduced sensitivity to penicillins, carbapenems 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 and Morrison *et al.,* 1997). Enterococci are also known to have acquired genetic determinants, which confer resistance to all classes of antimicrobial agents including chloramphenicol, tetracyclines, macrolides, streptogramins, lincosamides, β-lactams, aminoglycosides and most recently to glycopeptides (Panesso *et al.,* 2002).

2.10.1 Multi-Drug Resistance (MDR) in *Enterococcus* spp.

The emergence of resistance to multiple antimicrobial agents 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 one class of antibiotics and the 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 to be resistant to one or more commonly used drugs.

Peters *et al.* (2003) studied the resistance patterns of 118 selected *E. faecium* and *E. faecalis* strains to 13 antimicrobial active agents. The investigated strains were sensitive to ampicillin and amoxicillin/clavulanic acid. Only one *E. faecium* strain was resistant to penicillin, while all strains were sensitive to the glycopeptide antibiotics like vancomycin and teicoplanin. Most of the isolates were found resistant to tetracycline, quinupristin/dalfopristin and erythromycin.

Citak *et al.* (2005) stated that 177 enterococci isolated from 78 raw milk samples were showing high frequency of oxacillin, streptomycin and erythromycin resistance (95, 97 and 86%, respectively). Vancomycin and teicoplanin resistance was observed in 48% and 52% of *E. faecalis* isolates, respectively. The corresponding values were 26% and 33% for *E. faecium*. Resistance to vancomycin was significantly associated to erythromycin, rifampin, gentamicin, ampicillin and ceftriaxone resistance.

Messi *et al.* (2006) investigated antibiogram profile of 178 enterococci isolates of meat (59) and environmental origin (119) which revealed a low incidence of β-lactamic resistant strains, whereas high rate of resistance was observed for streptomycin (85.7% and 92.8%), kanamycin (79.7% and 96%) and gentamicin (85.1% and 91.7%) and an intermediate level resistant pattern emerged 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 isolates of enterococci from clinical and non-clinicl samples of equine origin for antibiogram profile against 19 antimicrobial agents

by disc diffusion method. Out of 267 isolates, 80.2% enterococci tested were resistant to vancomycin and 99.6% isolates were said to be MDR.

Kročko *et al.* (2011) reported that, out of 75 isolates of enterococci isolated from raw pork, raw beef and poultry, 15% were resistant to vancomycin and 15% to erythromycin, 27% to ampicillin, 25% to gentamicin and 56% to tetracycline. They also reported a higher prevalence of intermediate resistant isolates of pork and poultry to ampicillin (70% and 40%), gentamicin (66% and 40%), tetracycline (only pork 54%) and erythromycin (only pork 64%).

Enayati *et al*. (2015) observed the resistant pattern of enterococcus isolates recoverd from surface water to be 41.5, 27.1, 12.7, 6.8 and 1.7% to tetracycline, erythromycin, ampicillin, ciprofloxacin and chloramphenicol respectively. None of the *E. faecium* isolates were resistant to vancomycin, teicoplanin, linezolid, gentamicin and quinuspristin- dalfopristin.

Latha *et al*. (2016) reported that out of 60 enterococci isolates recoverd from Gomati river, 29 % isolates exhibit resistance to a macrolide, erythromycin and rifampicin in association with tetracycline.

2.10.2 Aminoglycoside resistance in *Enterococcus* spp.

Aminoglycosides have a broad spectrum activity and are considered to be lifesaving drugs in human therapy. Gentamicin, neomycin and streptomycin are clinically essential drugs however, their use in animal husbandry has been recommended by the United States Pharmacopeial Convention (2010) and Marshall and Levy, 2011). Aminoglycosides are bactericidal in action and have a broad spectrum activity against dreadful pathogens (Kotra *et al.,* 2000). They are also used in synergy with other antibiotics to overcome the effects of impermeability and are vital drugs in clinical settings (Eliopoulos and Moellering, 1996). Enterococci show resistantce to aminoglycosides mainly by four methods: reduced intake of aminoglycosides (Magnet

and Blanchard, 2005), 16S rRNA methylation (Cundliffe, 1989), mutations in ribosome (Carter *et al.,* 2000) and aminoglycoside inactivation (Ramirez and Tolmasky, 2010).

Aminoglycoside modifying enzymes (AMEs) are known to inactivate the aminoglycosides in both Gram-negative and Gram-positive bacteria. The AMEs consist of basically three enzymes viz., adenyltransferases (ANT), acetyltransferases (AAC) and phosphotransferases (APH) which catalyze the modification of the aminoglycosides by adenylylation, acetylation and phosphorylation, respectively in the bacterial cell (Ramirez and Tolmasky, 2010).

PCR can be used to discriminate between different kinds of genes encoding resistance to aminoglycosides like *aac(6')-Ie-aph(2")-Ia* and *aph(2˝)Ib* (Donabedian *et al.*, 2003), *aph(2˝)Ic* (Chow *et al*., 1997) and *aph(2˝)Id* (Tsai *et al*., 1998).

Thal *et al.* (1995) reported that 18 enterococci isolates obtained from 17 South Eastern Michigan supermarkets showed ampicillin resistance in 12 (67%), HLGR in the absence of streptomycin resistance occurred in two (11%) isolates, HLGR with high-level streptomycin resistance in two (11%) and high-level streptomycin (HLSR) resistance in the absence of HLGR in four (22%) isolates.

Jackson *et al.* (2005) reported that 187 isolates of enterococci derived from 444 samples of swine origin exhibited high level resistance to gentamicin (MIC 500 μg/ml), kanamycin (MIC 500 μg/ml), or streptomycin (MIC 1000 μg/ml). Eight aminoglycoside resistance genes were detected by using PCR, most frequent was *ant*(*6*)-*Ia* and *aac*(*6′*)-*Ii* gene from *E. faecium* isolates.

Kaçmaz and Aksoy (2005) reported that 264 consecutive enterococcal isolates recoverd from hospitals in Turkey showed greater resistance to aminoglycosides. Out of 264 isolates, high aminoglycoside resistance was found in 16% *E. faecalis* and 88% of *E. faecium* for gentamicin, 35% and 44%, respectively for streptomycin.

Mendiratta *et al.* (2008) reported that, out of 150 isolates recoverd from the clinical samples, 85.5% were *E. faecalis* and 14.7% were *E. faecium*. Forty six percent of isolates showed HLAR and most of the isolates showed MDR.

2.10.3 β-lactamase resistance (*blaZ*) in *Enterococcus* spp.

Complete or relative resistance to β-lactams is a characteristic feature of the genus *Enterococcus*. *β*-lactam antibiotics act by inhibiting the cell-wall synthesis. Penicillin-binding proteins (PBPs) that are involved in the synthesis and assembly of the peptidoglycan layer in the cell wall are the targets for β-lactam antibiotics. Intrinsic resistance towards β-lactam antibiotics in enterococci is due to low affinity of PBPs for the β-lactam agents. This resistance differs between different β-lactams, with penicillins having the most activity against enterococci, carbepenems having slightly less activity and with cephalosporins having the least activity (Fontana *et al*., 1990).

In oraganisms like *Staphylococcus*, β-lactamase production is inducible in nature, whereas in enterococci β-lactamase production is constitutive (Zscheck and Murray, 1991). The first published data of β-lactamase in enterococci was in 1983, when a research lab in Houston, Texas reported that a strain of *E. faecalis* was isolated (HH22) which produced *β*-lactamase enzyme (Murray and Mederski-Samaroj, 1983). A single cell and even a small number of cells may not produce a sufficient amount of enzyme to inactivate the antibiotic and will often appear susceptible; a large number of bacteria collectively produce more enzyme, can inactivate the antibiotic and thus test resistant. Several β-lactamase tests, including cefinase disks (containing the chromogenic cephalosporin nitrocefin), β-Lactam (Remel) and DrySlide Beta-Lactamase (Difco Laboratories) have been used successfully in identifying the β-lactamase production (Meszaros *et al.,* 1991). PCR based genotypic detection of β-lactamase production in *Enterococcus* spp. by targeting *blaZ* gene was developed (Tomayko *et al.*, 1996).

Tomayko *et al.* (1996) investigated 10 β-lactamase-producing *E. faecalis* for the presence of the staphylococcal β-lactamase repressor and antirepressor genes. They reported that 4 isolates, previously shown to be unrelated to each other by PFGE analysis, were positive for *blaZ* gene by PCR. Six isolates, previously shown to be clonally related, were negative for both repressor and anti-repressor genes by PCR.

2.10.4 Vancomycin resistance in *Enterococcus* spp.

Vancomycin has proved to be active against most Gram-positive pathogens and is used in the treatments of infections due to Staphylococci, Streptococci, Enterococci, Clostridia and Corynebacteria (Arthur *et al.*, 1996a). Gram-negative bacteria are, in general, resistant to glycopeptides because these antibiotics are unable to cross the outer cell envelope. Among glycopeptide family, vancomycin and teicoplanin are the only two currently in clinical practice.

2.10.4.1 Vancomycin resistance mechanisms:

VRE are often compounded by the use of antibiotics in animal farms as growth promoters. A link between the use of avoparcin as a growth promoter in poultry and swine farms and an increased occurrence of VRE in humans is well documented (Aarestrup, 1995; Klare *et al*., 1995; Kruse *et al*., 1999 and Wegener, 1999) and so is the evidence for transmission of VRE from animals to humans (Stobberingh *et al*., 1999).

The first isolates of glycopeptide resistant enterococci (GRE) were reported by investigators in the UK in 1986 (Leclercq *et al.,* 1988). Resistance to glycopeptides in enterococci, as understood to date, is phenotypically and genotypically heterogeneous. Six glycopeptide resistance phenotypes *vanA, vanB, vanC, vanD, vanE* and *vanG*, have been described in enterococci; the first two types are the most clinically relevant (Patel, 2005 and Taneja *et al.*, 2006a).

Laboratory experiments have achieved the transfer of high-level vancomycin resistance from enterococci to *Staphylococcus aureus* (Noble *et al.,* 1992). Vancomycin resistance has also been transferred in vitro by conjugation or transformation from enterococci to *Streptococcus sanguis*, *Lactococcus lactis*, *Streptococcus pyogenes* and *Listeria monocytogenes* (Power *et al.,* 1995 and Biavasco *et al.,* 1996).

2.10.4.1.1 *vanA* glycopeptide resistance

vanA phenotype glycopeptide resistance is characterized by acquired inducible high level resistance to both vancomycin (MICs 64mg/L->1000mg/L) and teicoplanin (MICs 16mg/L- 512mg/L) which has been reported in several enterococcal species (Arthur and Courvalin, 1993; Cercenado *et al.,* 1995; Dutka-Malen *et al.,* 1995 and Rosato *et al.,* 1995) and in certain VR *Staphylococcus aureus* (VRSA) isolates (CDC, 2002a and CDC 2002b). *vanA* is the most completely understood type of glycopeptide resistance (Arthur *et al.,* 1996b).

2.10.4.1.2 *vanB* glycopeptide resistance

vanB phenotype glycopeptide resistance is associated 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) (Patel, 2005 and Taneja *et al.,* 2006b). A few isolates with resistance also to teicoplanin have been described (Murray, 2000). The *vanB* gene cluster has homology to the *vanA* gene cluster but has been less well studied. It is found predominantly in *E. faecalis* and *E. faecium* (Arthur *et al.,* 1996a).

2.10.4.1.3 *vanC* glycopeptide resistance

vanC phenotype glycopeptide resistance is characterized by low-level vancomycin resistance (MIC 2mg/L-32mg/L) and susceptibility to teicoplanin (MIC 0.12mg/L-2mg/L) (Patel, 2005; Taneja *et al*., 2006b) and has been described as an intrinsic property of *E. gallinarum* and *E. casseliflavus/ flavescens* (Dutka-Malen *et al.,*

1992 and Navarro and Courvalin, 1994). Enterococci with *vanC* have lower affinity for vancomycin (Billot-Klein *et al.,* 1994).

2.10.4.2 Molecular detection of VRE

Since vancomycin resistance genes are transferable among different enterococci species or even among different genera of bacteria (Woodford *et al.,* 1995), the lack of prompt detection of enterococci may cause delays in attempting to prevent VRE colonization and infection. Conventional identification methods for enterococci require atleast 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 detection of enterococci in a variety of clinical specimens and can produce results in just a few hours. Many PCR-based methods for the specific detection of VRE have been reported (Dutka-Malen *et al.,* 1995; Clark *et al.,* 1998 and Ke *et al.,* 1999).

Clark *et al.* (1993) in USA examined 101 isolates for *vanA, vanB* and *vanC* phenotypes by PCR amplification of the vancomycin resistance genes and 99 were confirmed by production of specific 1030, 433 and 796bp PCR products, respectively.

Dutka-Malen *et al.* (1995) in France developed a PCR assay that permits simultaneous detection of GRE genotypes and identification of clinically relevant enterococci at species level (*E. faecium*, *E. faecalis*, *E. gallinarum* and *E. casseliflavus*). They found that this test offers a specific and rapid alternative to antibiogram profiles, in particular for detection of low-level resistance to vancomycin.

Cloning and characterization of the *vanD* and *vanE* genes will permit the development of PCR assays for the specific detection of these new types of glycopeptide resistance. Subsequent DNA sequencing of the PCR fragments gives information on the variability of glycopeptide resistance genes within each type of resistance class (Perichon *et al.,* 1997 and Fines *et al.,* 1999).

Mac *et al.* (2003) reported that out of 367 isolates recoverd from foods of animal origin, 21 isolates showed *vanA* gene. No *vanB* strain could be detected. The majority of the isolates possessed either *vanC1* or *vanC2*.

Messi *et al.* (2006) reported that out of 120 meat and 123 environmental samples analysed, 59 meat (35%) and 119 environmental enterococci isolates (26.5%) were found to be GRE. In particular, 10.7% meat isolates belong to the *vanA*, 10.7% to *vanB* and 16% to *vanC* phenotypes. Environmental samples presented 0.7% *vanA*, 14.5% *vanB* and 11.4% *vanC* strains.

Nishiyama *et al*. (2015) screened for presence of VRE in the aquatic environment, they screened sewage and urban river water samples from Miyazaki, Japan. Results showed 92% enterococci isolates containing *vanC2*/3 and were identified as *E. casseliflavus/gallinarum*, the remaining isolates were *E. faecium* (4%) and *E. faecalis* (4%) with *vanC2*/3.

Biswas *et al*. (2016a) reported that out of 500 *Enterococcus* isolates studied, a total of 60 VRE/VIE isolates were detected by MIC and were also confirmed to carry *vanA* or *vanB* genes by PCR.

Latha *et al*. (2016) reported that the frequency of VR *E. faecalis* was ranging from 22 to 100% from upstream to downstream in Gomati river along the Lucknow city landscape.

2.11 GENOTYPING

In general, typing of enterococci has been accomplished by analysis of proteins, biochemical profiles, antibiotic susceptibility and virulence patterns. Reliable molecular methods for determination of relatedness between bacterial isolates have become increasingly important to evaluate outbreaks and endemic situations with foodborne pathogens. Different methods like Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR (Blanco *et al*., 2017), Repetitive Extergenic Palindromic (REP) PCR

(Bedendo and Pignatari, 2000), PFGE- Pulsed-field gel electrophoresis (Mendez-Alvarez *et al.,* 1995 and Tenover, 1998), determination of 16S *rRNA* sequences (Monstein *et al.,* 1998 and Ke *et al.,* 1999), RFLP- Restriction Fragment Length Polymorphism (Scheidegger, 2009), MLST- Multilocus Sequence Typing (Maiden *et al.,* 1998; Enright and Spratt, 1999; Enright *et al.,* 2000 and Kalia *et al.,* 2001) and AFLP-Amplified Fragment Length polymorphisms (Savelkoul *et al.,* 1999 and Antonishyn *et al.,* 2000) are generally used for typing of *Enterococcus* spp. PCR based techniques like REP and ERIC PCR are accurate, rapid, reproducible, sensitive, specific and reliable diagnostics, which are used for determining different DNA fingerprints (Behzadi *et al*., 2015).

2.11.1 rep-PCR

Versalovic *et al*. (1991) developed genotyping methods on the basis of diversity in interspersed repetitive DNA sequence elements which is termed as rep-PCR or repREB-PCR (repetitive sequences REP-ERIC-BOX)-PCR. It has 3 different methods namely REP-PCR, ERIC-PCR and BOX-PCR based on 3 different repetitive elements.

2.11.1.1 ERIC-PCR

Among several PCR-based tools, the ERIC-PCR is a simple, sharp and cost effective genotyping technology for discriminating different types of strains. Indeed, ERICs are recognized as mobile DNA particles in association with Miniature Inverted Transposable Elements (MITEs) (Behandi Leung *et al*., 2004, Ranjbar *et al*., 2013a, Ranjbar *et al*., 2013b and Ranjbar *et al*., 2014).

Blanco *et al*. (2017) conducted genetic diversity studies on one *E. hirae* and 67 *E. faecalis* strains by ERIC-PCR. The banding patterns of DNA fragments were reported to be ranging from 236 to 3380bp. They also reported that *E. fecalis* strains were clustered into five major groups and one strain was unclustered whereas *E. hirae* strain was distantly related to the rest of the strains.

2.11.1.2 REP- PCR

The initial discovery of Repetitive Extragenic Palindromic (REP) elements occurred in the genomes of *Escherichia coli* and *Salmonella*. The application of REP-PCR to microbes has proven a discriminatory and reproducible tool for microbial subtype analyses and for microbial ecology investigations (Hiett and Seal, 2009).

Bedendo and Pignatari (2000) studied 8 *E. faecium* isolates from Stanford University and 12 from São Paulo Hospital by JB1-PCR, REP-PCR and PFGE. Among the isolates from Stanford University, 5 genotypes were defined by JB1-PCR, 7 by REP-PCR and 4 by PFGE. Among the isolates from São Paulo Hospital, 9 genotypes were identified by JB1-PCR, 6 by REP-PCR and 5 by PFGE.

CHAPTER - III

MATERIALS AND METHODS

The research work was carried out in the Department of Veterinary Public Health and Epidemiology, N.T.R. College of Veterinary Science (N.T.R. C.V.Sc.), Gannavaram.

3.1 BACTERIAL REFERENCE STRAINS AND DNA

The Microbial Type Culture Collection and Gene Bank (MTCC) reference strains of *E. faecalis* (MTCC439) and *E. gallinarum* (MTCC7049) were obtained from CSIR- Institute of Microbial Technology, Chandigarh. Revival of standard cultures was performed as per MTCC bacterial culture guide i.e. facultative anaerobic incubation at 37°C for 1-3 days till turbidity appears. Identification of the reference strain was re-confirmed by sub-culturing onto selective media and subjecting to standard biochemical tests. Further, it was tested at regular intervals of 15-30 days for viability, purity, morphological and biochemical characteristics.

3.2 MEDIA AND CHEMICALS

KF-streptococcus agar, KAA agar, 1% TTC solution, Brain Heart Infusion (BHI) broth, Brain heart infusion (BHI) agar, Mueller Hinton (MH) agar, Nutrient broth, egg yolk agar, skimmed milk and gelatin were procured from M/s. HiMedia Laboratories (Mumbai).

Analytical and molecular biology grade chemical reagents were obtained from M/s. Sisco Research Laboratories Pvt. Ltd. (SRL, Mumbai), M/s. Thermo Fisher Scientific India Pvt. Ltd. (Mumbai), M/s. Merck Specialities Pvt. Ltd. (Mumbai), M/s. SD Fine-Chem Limited (SDFCL, Mumbai), M/s. Merck Genie (Bengaluru) and from other National and international firms. Glassware used in this study were of Borosil and plasticware were of M/s. Tarsons Products Pvt. Ltd. (Kolkata) and other reputed

suppliers. Microbiological culture media, buffers and all other chemical reagents were prepared with autoclaved triple distilled water. The composition for media, buffers, stock and working solutions have been listed in Appendix-I and II.

3.3 OLIGONUCLEOTIDE PRIMERS

Oligonucleotide primers used in the present study were custom synthesized from M/s. Bioserve Biotechnologies Pvt. Ltd. (Hyderabad). They were reconstituted to a stock solution of 100 pmol/µl and to a working dilution of 10 pmol/µl with sterile nuclease free water and held at -20^{o}C.

3.4 SCIENTIFIC EQUIPMENT

The scientific equipment from national and international firms used in the study have been mentioned whenever necessary. Some of the important equipment used were Benchtop Incubator (New Brunswick Scientific, USA), Laminar Air flow (Thermo Scientific, USA), Refrigerated Centrifuge (Thermo Scientific, USA), Digital electronic balance (Sartorius, Germany), Nanodrop 200C (Thermo Scientific, USA), Biological deep freezer -20^{o}C (Vestfrost solutions), Thermal cycler (Eppendorf, USA), Horizontal electrophoresis unit (Atto, Japan) and Gel documentation System with Image Lab Software (BIO-RAD, USA).

3.5 SAMPLE COLLECTION

A total of 780 samples were collected including 234 food samples from poultry and quail; 324 food samples of animal origin (80 mutton, 56 raw pork, 54 fish, 52 cara beef and 82 milk from bulk milk centres; 85 faecal swabs (32 chicken cloacal swabs, 18 buffalo rectal swabs, 23 sheep rectal swabs and 12 pig rectal swabs); 25 water samples; 40 uterine discharges of cattle as well as 42 stool samples from veterinary students, 12 human diarrhoeic stool samples and 18 human urine samples from different places of Krishna district (Table-2 and 3). Samples were collected from slaughter houses, local meat vendors, clinical samples from local hospitals and diagnostic centers for over a

period lasted from September, 2018 to July, 2019. The flow chart of methodology of present study was depicted in Fig-1.

Table-2: Details of samples collected from animal sources

Type of samples	Source		No. of samples tested
	FOOD SAMPLES		
Chicken (173)	Chicken retail shops in and around Gannavaram		101
	Chicken retail shops in and around Gudiwada		72
Quail (61)	Livestock Farm Complex, NTR CVSc, Gannavaram		32
	Retail chicken shops in and around Gannavaram		29
Mutton (80)	Retail mutton shops in and around Gannavaram		52
	Retail mutton shops in and around Kankipadu		28
Pork (56)	Retail shops in and around Gannavaram		19
	Department of Livestock Products and Technology, Pork Processing Unit, NTR CVSc, Gannavaram		37
Fish (54)	Local fish market in and around Gannavaram		54
Carabeef (52)	Kabela in Vijayawada		32
	Slaughter house in Hanuman Junction		20
Milk from bulk milk collection centers (82)	Kesarapalli		16
	Gannavaram		10
	Atukuru		14
	Kankipadu		22
	Uppuluru		20
	ANIMAL FAECAL SWABS		
Faecal swabs (85)	**Chicken**	Poultry farm, LFC, N.T.R. C.V.Sc., Gannavaram	32
	Pig	Pig farm, LFC, N.T.R. C.V.Sc., Gannavaram	12
	Buffaloes	Cattle farm, LFC, N.T.R. C.V.Sc., Gannavaram	18
	Sheep	Sheep farm, LFC, N.T.R. C.V.Sc., Gannavaram	23
	ENVIRONMENTAL SAMPLES		
Water (25)	Different locations in Vijayawada		25
	MISCELLANEOUS		
Clinical samples (40)	Uterine discharges collected from cattle at Veterinary Clinical Complex, NTR CVSc, Gannavaram		40
	TOTAL		**708**

Table-3: Details of samples collected from human sources

S.NO	Type of samples	Source	No. of samples tested
1	Stool samples	Students of Gokul Hostel, N.T.R. C.V.Sc, Gannavaram	42
2	Human diarrhoeic stool samples	Microbiological laboratories in Vijayawada	12
3	Human urine samples	Microbiological laboratories in Gannavaram	18
		TOTAL	**72**
		GRAND TOTAL	**780**

3.5.1 Collection of raw meat and milk samples

Approximately 10g each of different fresh raw meat samples were collected in sterile polythene zip lock packs under aseptic conditions. Raw milk and water samples (10ml each) were collected in sterile test tubes with screw cap. Meat, milk and water samples were transferred immediately on to ice and transported to the Food Safety Laboratory, Department of Veterinary Public Health and Epidemiology, N.T.R. C.V.Sc., Gannavaram and processed within 24h of collection.

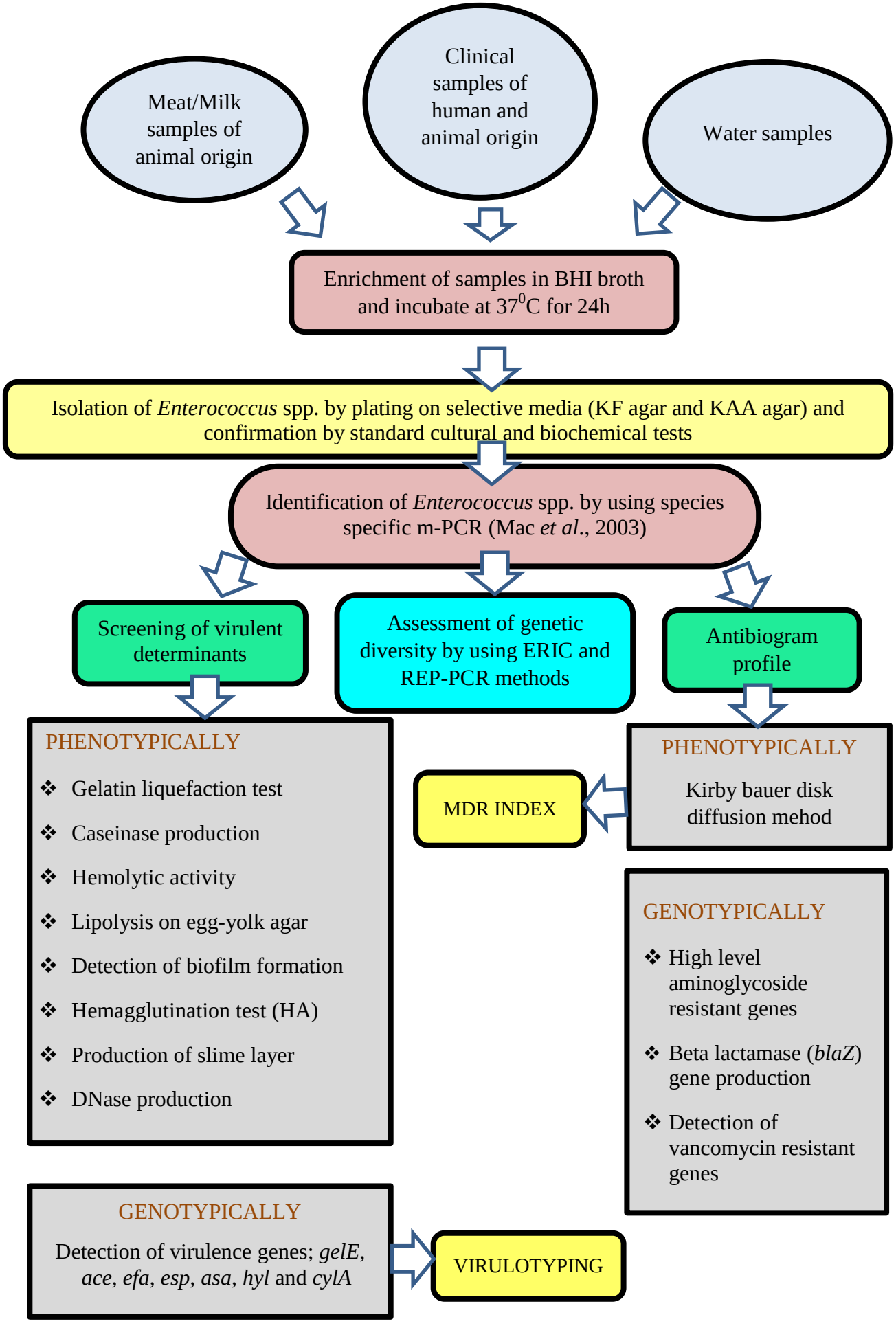
3.5.2 Collection of faecal swab samples

Sterile cotton tipped viscose swab (HiMedia, Mumbai) was inserted into rectum of human and animals or vent of the birds rotated gently against the mucosa for approximately 5-10 seconds, until covered with a uniform amount of faeces. Utmost care was taken in order to minimize contact with air, by transferring the faecal swabs to sterile tubes containing 10ml of phosphate buffered saline (PBS, pH 7.4) immediately after collection without any delay.

3.5.3 Collection of human stool and urine samples

Stool samples from veterinary students (attending clinical wards of N.T.R. C.V.Sc., Gannavaram) as well as human urine and diarrhoeic stool samples (from Rx clinical laboratories, Gannavaram) were collected (approximately 10g/ 10ml each) in sterile test tubes with screw cap under aseptic conditions.

Figure-1: Flow chart of methodology of study



3.6 ISOLATION AND IDENTIFICATION OF *ENTEROCOCCUS* SPP.

3.6.1 Cultural isolation

Ten grams each raw meat sample and human diarrhoeic stool samples collected from various sources were mixed with 90ml of BHI broth and homogenized within 24h of collection. Ten grams/10ml each of milk, water, uterine discharges, stool and urine samples are transferred into 90ml of BHI broth. Following homogenization, samples were incubated at 37ºC for 24h for enrichment. Enriched samples were streaked on KF-streptococcus agar plates and incubated at 37ºC for 48h. Deep red colour colonies indicate *Enterococcus* spp. Milk samples were selectively cultured on Kanamycin aesculin azide agar incubated at 37^0C for 24h. Transparent colonies surrounded with black haloes are characteristic of *Enterococcus* spp.

3.6.2 Presumptive identification by Gram's staining

Typical colonies of *Enterococcus* spp. on KF-streptococcus agar and KAA agar were further processed for presumptive identification by Gram's staining, which showed the presence of Gram positive cocci either single or in pairs or short chains.

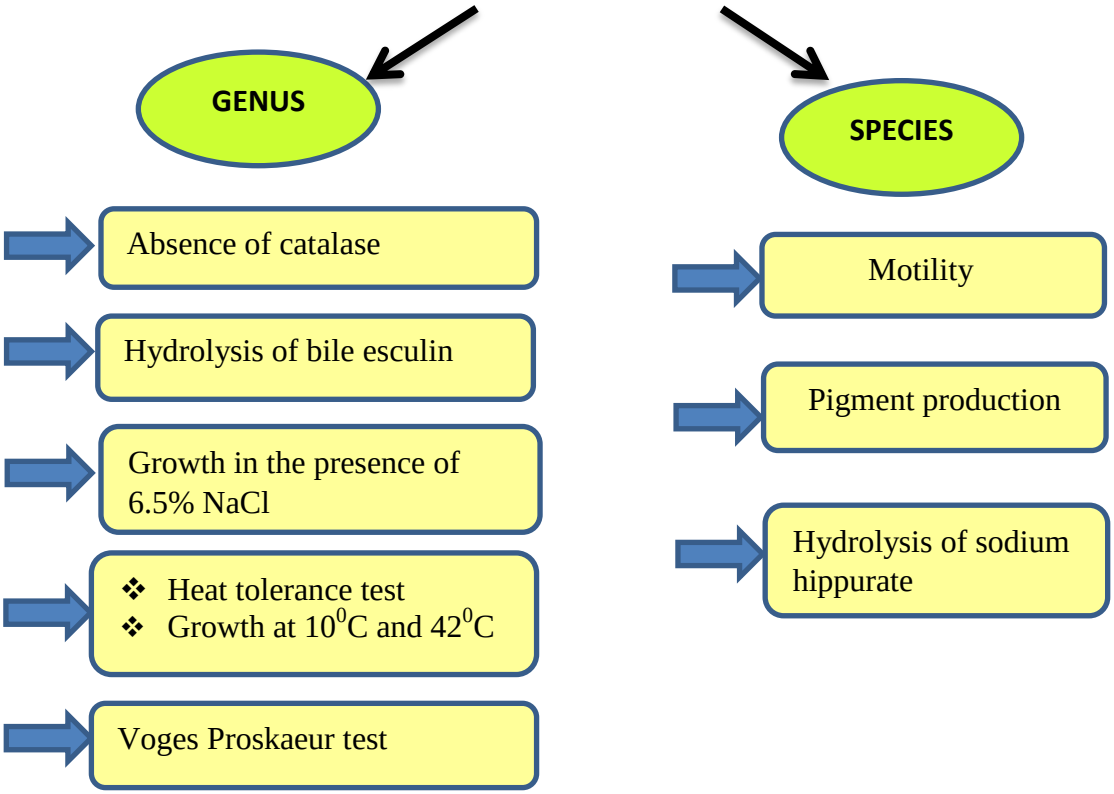
3.6.3 Biochemical characterization

For confirmation of *Enterococcus* spp., the biochemical tests conducted were catalase test, aesculin hydrolysis, hippurate hydrolysis, oxidase test and Voges Proskaeur test. The procedures used for the conduction of biochemical tests were given in detail in Appendix-II.

3.6.4 Genus and species identification by phenotypic methods

The genus *Enterococcus* and different species were confirmed phenotypically as given in Fig-2 (Liassine *et al*., 1998, Cetinkaya *et al*., 2000 and Forbes *et al*., 2007).

Fig-2 GENUS AND SPECIES IDENTIFICATION OF *ENTEROCOCCUS* SPP. BY PHENOTYPIC METHODS



3.7 MOLECULAR DETECTION OF THE GENUS *ENTEROCOCCUS*

Molecular confirmation of *Enterococcus* was done by genus-specific PCR as described by Poyart *et al*. (2000) targeting *sodA* gene. PCR reaction was carried out in 25µl optimized reaction mixture (Table-4) under standardized thermal cycling conditions (Table-5).

Table-4 Optimized PCR reaction mixture for amplification of genus *Enterococcus*

Components	Volume/Reaction
10x Taq buffer	2.75µl
25mM $MgCl_2$	1.00µl
10mM dNTPs	0.50µl
Forward primer (10 pmol/µl)	1.5µl
Reverse primer (10 pmol/µl)	1.5µl
Taq DNA polymerase (1U/µl)	1.00µl
Template DNA (50ng/µl)	1.50µl
Nuclease free water	15.25µl
Total	**25µl**

Table-5 Primers and standardized thermal cycling conditions used for detection of genus *Enterococcus*

Primer	Primer sequence (5^1 -3^1) and thermal cycling conditions	Size (bp)	Reference
SodA	F-CCITAYICITAYGAYGCIYTIGARCC R-ARRTARTAIGCRTGYTCCCAIACRTC Initial denaturation at 94^0C for 3 min Denaturation at 95^0C for 30sec Annealing at 37^0C for 60 sec (30cycles) Extension at 72^0C for 60 sec Final cycle elongation at 72^0C for 10 min	**438**	Poyart *et al*. (2000)

In the above listed primer sequence I- A/T/G/C (based on spp.)

R- A or G

Y- C or T

3.8 DNA EXTRACTION BY WHOLE CELL HEAT-LYSIS METHOD (BOILING AND SNAP CHILL METHOD)

3.8.1 DNA extraction from colonies

Loopful of 24h growth culture was suspended in 150µl of nuclease free water in a 0.5ml microcentrifuge tube. After mixing properly, the tubes were heated to 100°C in water bath for 15min and immediately placed on ice (-20°C). After 20min the bacterial lysate was centrifuged at 13,000rpm for 5min and the supernatant was used as DNA template for PCR assays (Siqueira *et al.,* 2002).

3.8.2 Measurement of DNA concentration and purity

The concentrations of DNA were measured with Nanodrop and adjusted to 50ng/µl for further PCR studies. Pure DNA samples (with an optical density ratio of 1.8 to 2 at 260/280nm) were stored at -20^0C, until further use.

3.9 MOLECULAR DETECTION OF *ENTEROCOCCUS* SPP.

Molecular detection of four *Enterococcus* spp. was done by using one m-PCR reaction for *E. gallinarum* and *E. casseliflavus* and two uniplex PCR reactions (I and II) for *E. faecalis* and *E. faecium*, respectively targeting *sodA* (genus specific partial sequence of superoxide dismutase) . PCR reaction was carried out in 25µl optimized reaction mixture (Table-6). Oligonucleotide primers and standardized thermal cycling conditions used for detection of four *Enterococcus* spp. (Table-7) by PCR was carried out the method described by as per Dutka-Malen *et al*. (1995) and Jackson *et al*. (2004).

Table-6 Optimized PCR reaction mixture for amplification of *Enterococcus* spp.

	Volume/reaction		
Components	**m-PCR I (*E. gallinarum and E. casseliflavus*)**	**Uniplex PCR-I (*E. faecalis*)**	**Uniplex PCR-II (*E. faecium*)**
10x Taq buffer	2.50µl	2.75µl	2.50µl
25mM $MgCl_2$	1.50µl	1.00µl	1.5µl
10mM dNTPs	1.00µl	0.50µl	0.50µl
Forward primer (10 pmol/µl)	2 X 0.75µl	1.5µl	1.00µl
Reverse primer (10 pmol/µl)	2 X 0.75µl	1.5µl	1.00µl
Taq DNA polymerase (1U/µl)	1.00µl	1.00µl	1.00µl
Template DNA (50ng/µl)	2.00µl	1.50µl	1.00µl
Nuclease free water	14.00µl	15.25µl	16.50µl
Total	**25µl**	**25µl**	**25µl**

3.10 POST PCR VALIDATION BY ANALYTICAL AGAROSE GEL ELECTROPHORESIS

Gel electrophoresis was carried out to analyze the PCR amplicons (Sambrook and Russell, 2001). Agarose gel (1.5%) was prepared in 1X TAE (Tris acetate EDTA) buffer with ethidium bromide @ 0.5µg/ml. The molten agarose was poured in to a gel casting tray fitted with acrylic comb. The tray containing solidified gel was then placed in a submarine horizontal electrophoresis unit filled with 1X TAE buffer up to a level of one mm above the gel surface and the comb was then taken out carefully. About 10µl of PCR product was mixed with 2µl of 6X gel loading dye (Fermentas, Banglore) and slowly loaded into the wells. Three µl of 100 bp DNA ladder was loaded as molecular weight marker (Genei^{TM}, Banglore) in one well. Electrophoresis was performed at 5V/cm for approximately one hour or until the dye has migrated to two-third distance (75-80%) down the gel.

TABLE-7: Primers and standardized thermal cycling conditions used for detection of different *Enterococcus* spp.

Primer target gene	Primer sequence	Size (bp)	PCR conditions	Reference
***E. faecium* (*sodA*)**	F- 5'-GCAAGGCTTCTTAGAGA-3' R- 5'-CATCGTGTAAGCTAACTTC-3'	550	Initial denaturation at 94^0C for 4 min Denaturation at 95^0C for 30sec Annealing at 54^0C for 30 sec Extension at 72^0C for 30 sec (30cycles) Final cycle elongation at 72^0C for 10 min	Dutka-Malen *et al*. (1995)
***E. faecalis* (*sodA*)**	F: 5′-ACTTATGTGACTAACTTAACC-3′ R: 5′-TAATGGTGAATCTTGGTTTGG-3′	360	Initial denaturation at 95^0C for 3 min Denaturation at 95^0C for 30sec Annealing at 51^0C for 30 sec Extension at 72^0C for 50 sec (30cycles) Final cycle elongation at 72^0C for 8 min	Jackson *et al*. (2004)
***E. gallinarum* (*sodA*)**	F: 5′-TTACTTGCTGATTTTGATTCG-3′ R: 5′-TGAATTCTTCTTTGAAATCAG-3′	173	Initial denaturation at 95^0C for 4 min Denaturation at 95^0C for 30sec Annealing at 55^0C for 60 sec Extension at 72^0C for 60 sec (30cycles) Final cycle elongation at 72^0C for 7 min	
***E. casseliflavus* (*sodA*)**	F: 5′-TCCTGAATTAGGTGAAAAAAC-3′ R: 5′-GCTAGTTTACCGTCTTTAACG-3′	288		

The bands were visualized with ethidium bromide fluorescence at 300nm wave length using a UV transilluminator (BIO-RAD Gel Documentation system, USA) and the images were photographed using the supplied Image Lab software. Amplified PCR product size was determined by comparing with the standard molecular weight marker.

3.11 DETECTION OF PUTATIVE VIRULENCE GENES IN *ENTEROCOCCUS* SPP.

3.11.1 Phenotypic detection of virulence factors in *Enterococcus* spp.

The following phenotypic methods were used for detection of virulence factors in *Enterococcus* spp. (Aberna and Prabakaran, 2011; Winn, 2006 and Forbes *et al*., 2007).

3.11.1.1 Gelatin liquefaction test

Gelatinase production was detected by stab inoculating the test strain on 3% gelatin nutrient broth and incubated at 37ºC for 24h followed by refrigeration at 4ºC for half an hour. Liquefaction of gelatin was considered as positive.

3.11.1.2 Caseinase production

Casein hydrolysis was detected on MHA containing 3% skimmed milk. Plates were streaked with test strains followed by incubation at 37^0C for 24h. The presence of a transparent zone around the colonies indicates caseinase activity.

3.11.1.3 Hemolytic activity of enterococci

It was assessed on blood agar plates prepared with MHA containing 5% defibrinated sheep blood and incubated for 24h at 37^0C. Zone of hemolysis around the colonies indicates positive reaction.

3.11.1.4 Lipolysis on egg-yolk agar

Egg yolk agar was used for lipase production. The test organism was spot inoculated on the medium and incubated at 37^0C for 24 to 48h. Thin iridescent pearly

layer overlying the colonies is indicative of positive result with a confined opalescence in the medium, which was seen when the colonies were scraped off.

3.11.1.5 Detection of biofilm formation by microtiter plate (MTP) assay

The test strains were grown overnight at 37ºC in BHI broth with 0.25% glucose. Culture was diluted to 1:20 in the same medium. About 200µl of this suspension was used to inoculate 96 well sterile polystyrene microtitre plates. After static incubation at 37^0C for 24h, wells were washed with PBS, dried in inverted position and stained with 1% crystal violet for 15 min. The wells were rinsed once more and solubilized in 200 µl ethanol/acetone (80:20v/v). The absorbance at 630nm (A630) was determined using MTP reader. Biofilm formation was scored as non-biofilm forming (-), weak (+), moderate (++), strong(+++) corresponding to the A630 values ≤0.120, 1- ≤0.120-0.240 and >0.240, respectively (Hashem *et al*., 2017).

3.11.1.6 Hemagglutination test (HA)

Enterococci were grown on BHI agar supplemented with 10% sheep blood. A loopful of bacteria was mixed on a glass slide with 25µl of a 3% suspension of sheep/ rabbit/ human group A/ human group O or human group B erythrocytes. Clearing of the suspension and clump formation indicates positive reaction. After 5min at room temperature, results were recorded as positive or negative.

3.11.1.7 Production of slime layer

BHI agar supplemented with 5% sucrose was used to determine the ability of *Enterococcus* spp. to produce extracellular polysaccharide on the agar. Test strains grown in Todd Hewitt broth (HiMedia, Mumbai, India) was used as the inoculum. The colonies appear as mucoidal, runny or slimy due to production of polysaccharide.

3.11.1.8 DNase production

Test strains were inoculated on DNAse agar (HiMedia, Mumbai). When DNA is hydrolysed the methyl green indicator released combines with highly polymerized DNA at a pH of 7.5, turning the medium from colorless to yellow around the test organism.

3.11.2 Genotypic/Molecular detection of putative genes in *Enterococcus* spp.

All the confirmed enterococci isolates were subjected to 2 m-PCR assays for the detection of seven putative virulence genes (*gelE , ace, efaA, esp, asa, cyl*A and *hyl*) as described by Eaton and Gasson (2001), Creti *et al*. (2004) and Vankerckhoven *et al*. (2004). PCR reaction was carried out in 25µl optimized reaction mixture (Table-8). Primer sequences and standardized thermal cycling conditions for detection of virulent genes were described under Table-9. Band pattern was visualized as per 3.10.

Table-8 Optimized PCR reaction mixture for amplification of virulence genes in *Enterococcus* spp.

Components	m-PCR (I and II)
10x Taq buffer	3.00µl
25mM $MgCl_2$	1.50µl
10mM dNTPs	1.00µl
Forward primer (10 pmol/µl)	0.75µl X 3
Reverse primer (10 pmol/µl)	0.75µl X 3
Taq DNA polymerase	1.00µl
Template DNA (50ng/µl)	2.00µl
Nuclease free water	12.00µl
Total	**25µl**

TABLE-9: Primers and standardized thermal cycling conditions used for detection of putative virulence genes in *Enterococcus* spp.

Primer target gene	Sequence forward /reverse	Size (bp)	Reference	PCR conditions
gelE	F: 5′-ACCCCGTATCATTGGTTT-3′ R: 5′-ACGCATTGCTTTTCCATC-3′	419	Eaton and Gasson (2001)	Initial denaturation at 95^{0}C for 3 min Denaturation at 95^{0}C for 30sec Annealing at 51^{0}C for 30 sec } 30 cycles Extension at 72^{0}C for 50 sec Final cycle elongation at 72^{0}C for 8 min
esp	F:5′-TTGCTAATGCTAGTCCACGACC-3′ R: 5′-GCGTCAACACTTGCATTGCCGAA-3′	933		
efaA	F: 5′-GCCAATTGGGACAGACCCTC-3′ R: 5′-CGCCTTCTGTTCCTTCTTTGGC-3′	688	Creti *et al*. (2004)	
ace	F: 5′-GGAATGACCGAGAACGATGGC-3′ R: 5′-GCTTGATGTTGGCCTGCTTCCG-3′	616		
asa	F: 5′-GCACGCTATTACGAACTATGA-3′ R: 5′-TAAGAAAGAACATCACCACGA-3′	375	Vankerckhoven *et al*. (2004)	Initial denaturation at 95^{0}C for 15 min Denaturation at 95^{0}C for 60sec Annealing at 51^{0}C for 60 sec } 30 cycles Extension at 72^{0}C for 60 sec Final cycle elongation at 72^{0}C for 10 min
cylA	F: 5′-ACTCGGGGATTGATAGGC-3′ R: 5′-GCTGCTAAAGCTGCGCTT-3′	600		
hyl	F: 5′-ACAGAAGAGCTGCAGGAAATG-3′ R: 5′-GACTGACGTCCAAGTTTCCAA-3′	200		

3.12 ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antibiogram of *Enterococcus* spp. was done by Kirby- Bauer disc diffusion on MHA (Bauer *et al*., 1966 and CLSI, 2008).

3.12.1 Preparation of inoculum

Using a sterile loop, 3-4 similar looking colonies on selective agar were picked and transferred into a tube containing MH broth. The tube was incubated at 37ºC until its turbidity matched 0.5 Mc Farland's standard. After adjusting the turbidity, a sterile non-toxic cotton swab was dipped into the tube. The swab was rotated firmly against the upper inside wall of the tube to expel excess fluid.

Dried MHA plates were then inoculated by streaking the entire agar surface with the swab three times, turning the plate at 60º angle between each streaking for even distribution of inoculum. Finally the rim of the agar plate was swabbed. The inoculated plate was allowed to dry for 10-15min with lid in place before applying antibiotic discs.

3.12.2 Testing of antibiotics

Commercially available antibiotic discs were placed on the surface of MHA plates using sterile forceps. The discs were placed evenly at a distance not less than 24 mm apart and gently pressed on the agar surface for uniform contact. The plates were then inverted and incubated at 37ºC overnight. Antimicrobial susceptibility test discs used in the present study, their concentration and inhibition zone diameters used for inferring resistance were mentioned in Table-10.

Table-10: Interpretation chart for antibiotic sensitivity/resistance pattern of *Enterococcus* spp.

Antibiotic disc		Symbol	Disc content	Interpretative criteria		
				Sensitive	Intermediate	Resistant
1.	Ampicillin	AMP	10µg	≥ 17 mm	-	≤ 16 mm
2.	Ceftazidime	CAZ	30µg	≥ 21 mm	18-20 mm	≤ 17 mm
3.	Chloramphenicol	C	30µg	≥ 18 mm	13-17 mm	≤ 12 mm
4.	Ciprofloxacin	CIP	5µg	≥ 21 mm	16-20 mm	≤ 15 mm
5.	Erythromycin	E	15µg	≥ 23 mm	14-22 mm	≤ 13 mm
6.	Gentamicin	GEN	10µg	≥ 15 mm	13-14 mm	≤ 12 mm
7.	Linezolid	LZ	30µg	≥ 23 mm	21-22 mm	≤ 20 mm
8.	Nitrofurantoin	NIT	300µg	≥ 17 mm	15-16 mm	≤ 14 mm
9.	Penicillin-G	P	1U	≥ 15 mm	-	≤ 14 mm
10.	Piperacillin	PI	100µg	≥ 21 mm	15-20 mm	≤ 14 mm
11	Polymixin	PB	300U	≥ 17 mm	-	≤ 11 mm
12.	Rifampicin	RIF	5µg	≥ 20 mm	17-19 mm	≤ 16 mm
13.	Streptomycin	S	30µg	≥ 10 mm	7-9 mm	≤ 6 mm
14.	Teicoplanin	TEI	30µg	≥ 14mm	11-13 mm	≤ 10 mm
15.	Tetracycline	TE	30µg	≥ 15 mm	12-14 mm	≤ 11 mm
16.	Vancomycin	VA	30µg	≥ 17 mm	15-16 mm	≤ 14 mm

3.13 MOLECULAR DETECTION OF RESISTANCE GENES IN *ENTEROCOCCUS* SPP.

Genotypic methods like PCR was used to detect the antibiotic resistance towards enterococcus isolates at molecular level. Gene markers majorly high level aminogycoside resistance genes, betalactamase gene and vancomycin resistance genes were used for detection of antimicrobial resistance in *Enterococcus* spp.

3.13.1 Molecular detection of high HLAR genes in *Enterococcus* spp.

The *Enterococcus* isolates which exhibited phenotypic resistance to either gentamicin/streptomycin were subjected to HLAR phenotypic detection. All the isolates which exhibited resistance for either of the aminoglycosides i.e. gentamicin/ kanamycin/ streptomycin/ amikacin were selected for molecular detection of aminoglycoside resistance genes. The protocol for PCR amplification was followed according to Donabedian *et al*. (2003). It consists of one uniplex for "*aac(6')Ie-aph(2")Ia* gene and one multiplex for *aph(2")Ib*, *aph(2")Ic* and *aph(2")Id* genes. PCR was optimized in 25µl reaction mixture (Table-11). The primer sequences and cycling conditions for the 4 aminoglycoside resistance genes are enlisted in Table-12. The amplified PCR products were examined on 1.5% agarose gel in 1X TAE buffer. Band pattern was visualized as per 3.10.

Table-11 Optimized reaction mixtures for PCR reaction targeting aminoglycoside resistance genes

	Volume/ reaction	
Components	**Multiplex PCR I**	**Uniplex PCR I**
10x Taq buffer	3.00µl	2.75µl
25mM $MgCl_2$	1.50µl	1.00µl
10mM dNTPs	1.00µl	0.50µl
Forward primer (10 pmol/µl)	3 X 0.5µl	1.5µl
Reverse primer (10 pmol/µl)	3 X 0.5µl	1.5µl
Taq DNA polymerase (1U/µl)	1.00µl	1.00µl
Template DNA (50ng/µl)	2.00µl	1.50µl
Nuclease free water	13.50µl	15.25µl
Total	**25µl**	**25µl**

3.13.2 Molecular detection of beta-lactamase (*blaZ*) genes in *Enterococcus* spp.

A single uniplex PCR assay (Martineau *et al*., 2000) was standardized for the detection of β-lactamase gene in enterococci. PCR was optimized in 25 µl reaction mixture (Table-13). Primer sequence and standardized thermal cycling conditions used for detection of betalactamase gene (*blaZ*) were given in Table-15. Band pattern was visualized as per 3.10.

Table-13: Optimized reaction mixture for PCR targeting β-lactamase (*blaZ*) gene in *Enterococcus* spp.

Components	Uniplex PCR
10x Taq buffer	2.50µl
25mM $MgCl_2$	1.50µl
10mM dNTPs	0.50µl
Forward primer (10 pmol/µl)	1.00µl
Reverse primer (10 pmol/µl)	1.00µl
Taq DNA polymerase (1U/µl)	1.00µl
Template DNA (50ng/µl)	1.00µl
Nuclease free water	16.50µl
Total	**25µl**

3.13.3 Molecular detection of vancomycin resistance genes in *Enterococcus* spp.

A single m-PCR assay (Klare and Witte, 1997) was standardized for the detection of vancomycin resistant genes in enterococci. PCR was optimized in 25 µl reaction mixture (Table-14). Primer sequence and standardized thermal cycling conditions used for detection of vancomycin resistant genes were given in Table-15. Band pattern was visualized as per 3.10.

Table-14 Optimized PCR reaction mixture for amplification of VR genes in *Enterococcus* spp.

Components	m-PCR
10x PCR buffer	2.50µl
25mM $MgCl_2$	0.50µl
10 mM dNTPs	1.00µl
Forward primer (10 pmol/µl)	1.50µl
Reverse primer (10 pmol/µl)	1.50µl
Taq DNA polymerase (1U/µl)	1.00µl
Template DNA (50 ng/µl)	2.00µl
Nuclease free water	15.00µl
Total	**25µl**

TABLE-15: Primers and standardized thermal cycling conditions used for detection of *blaZ* and VRE gene in *Enterococcus* spp.

Primer target gene	Sequence forward/reverse	Size (bp)	PCR conditions	Reference
blaZ	F-ACTTCAACACCTGCTGCTTTC R-TGACCACTTTTATCAGCAACC	173	Initial denaturation at 95^{0}C for 3 min Denaturation at 95^{0}C for 30sec Annealing at 54^{0}C for 30 sec Extension at 72^{0}C for 30 sec (30 cycles) Final cycle elongation at 72^{0}C for 4 min	Martineau *et al*. (2000)
vanA	5'-TCT GCA ATA GAG ATA GCC GC-3' 5'-GG AGT AGC TAT CCC AGC ATT-3'	377	Initial denaturation at 94^{0}C for 4 min Denaturation at 95^{0}C for 30sec Annealing at 54^{0}C for 30 sec Extension at 72^{0}C for 30 sec (30 cycles) Final cycle elongation at 72^{0}C for 10 min	Klare and Witte (1997)
vanB	5'-CAT CGC CGT CCC CGA ATT TCA AA-3' 5'-GAT GCG GAA GAT ACC GTG GCT-3'	298		
vanC1	5'-GAC CCG CTG AAA TAT GAA G-3' 5'-CGG CTT GAT AAA GAT CGG G-3'	438		
vanC2	5'-CTC CTA CGA TTC TCT TG-3' 5'-CGA GCA AGA CCT TTA AG-3'	430		

TABLE-12: Primers and standardized thermal cycling conditions used for detection of aminoglycoside resistance genes in *Enterococcus* spp.

Primer target gene	Primer Sequence	PCR conditions	Size (bp)	Reference
aac(6´)Ia **aph(2´)Ie**	F-GAGCAATAAGGGCATACCAAAAATC R-CCGTGCATTTGTCTTAAAAAACTGG	Initial denaturation at 95^{0}C for 3 min Denaturation at 94°C for 30 s, Annealing at 56°C for30s, Extension at 72°C for 2 min } 35 cycles Final cycle elongation at 72^{0}C for 8 min	505	Donabeadian *et al.* (2003)
aph(2´)Ib	F- GCAAATGGCACAGTATAATATGC R- CTGAATTGCTACAAACACAAGC	Initial denaturation at 95^{0}C for 3 min Denaturation at 94°C for 30 s, Annealing at 56°C for 45 s, Extension at 72°C for 2 min } 35 cycles Final cycle elongation at 72^{0}C for 8 min	958	
aph(2´)Ic	F- GAAGTGATGGAAATCCCTTCGTG R- GCTCTAACCCTTCAGAAATCCAG		627	
aph(2´)Id	F- GGTGGTTTTTACAGGAATGCCATC R- CCTCTTCATACCAATCCATATAAC		642	

3.14 ASSESSMENT OF GENETIC DIVERSITY OF VRE

ERIC-PCR and REP-PCR were performed in order to assess the genetic relatedness of VRE isolates from different sources and to characterize them at species level.

3.14.1 Genotyping of VRE by ERIC-PCR

VRE isolates from different sources were fingerprinted using ERIC-PCR assay as described by Blanco *et al*. (2017) with minor modifications in order to obtain better band pattern. ERIC-1 (5^1-ATGTAAGCTCCTGGGGATTCAC-3^1) and ERIC-2 (5^1-AAGTAAGTGACTGGGGTGAGCG-3^1) primer pair was used for the amplification of conserved ERIC sequences in the chromosomal DNA of VRE isolates. ERIC-PCR was carried out in 25 µl optimized reaction mixture (Table-16) under standardized thermal cycling conditions (Table-17). PCR products were subjected to 1.5% agarose gel electrophoresis and visualized as per 3.10.

3.14.2 Analysis of ERIC-PCR fingerprinting patterns for VRE

ERIC-PCR fingerprints (banding patterns) of all the VRE isolates were compared by visual inspection. The position of bands on each lane and each gel were compared using 100 bp DNA ladder as an external reference standard. Presence of a particular band in a strain was coded as 1 and the absence of that particular band was coded as 0 in a binary matrix. The binary data was analysed using dollop programme of phylip version 3.6 (Felsenstein, 1989) software with default options. Dendrograms were constructed and analyzed separately for the 4 VRE species to establish genetic relationship or degree of similarity between isolates from different sources.

3.14.3 Genotyping of VRE by REP-PCR

Enterococcus isolates showing vancomycin resistant genes from different sources were subjected to REP-PCR fingerprinting using single oligonucleotide primer $(GTG)_5$ (5^1 GTGGTGGTGGTGGTG 3^1) as described by Bedendo and Pignatari (2000)

with slight modifications. PCR reactions were optimized in 25 µl volume reaction mixture (Table-16) under standardized thermal cycling conditions given in Table-17. PCR products were subjected to 1.5% agarose gel electrophoresis and visualized as per 3.10.

3.14.4 Analysis of REP-PCR fingerprinting patterns of VRE

The REP-PCR fingerprints (banding patterns) were compared visually with 100 bp DNA ladder and transformed into a binary character matrix ('1' for the presence and '0' for the absence of a band at a particular position). The binary data was analysed using dollop programme of phylip version 3.6 (Felsenstein, 1989) software with default options. Dendrograms were constructed for the four *Enterococcus* species separately to establish genetic diversity or relatedness among the VRE isolates.

Table -16: Optimized PCR reaction mixture for ERIC-PCR and REP-PCR for VRE

	Volume/reaction	
Components	**ERIC-PCR**	**REP-PCR**
10x Taq buffer	2.50µl	3.00µl
25mM $MgCl_2$	1.50µl	1.00µl
10mM dNTPs	0.50µl	0.50µl
Forward primer (10 pmol/µl)	1.00µl	$(GTG)_5$ primer 2.00µl
Reverse primer (10 pmol/µl)	1.00µl	
Taq DNA polymerase (1U/µl)	1.00µl	1.00µl
Template DNA (50 ng/µl)	2.00µl	2.00µl
Nuclease free water	15.50µl	15.50µl
Total	**25µl**	**25µl**

Table-17: Standardized thermal cycling parameters for ERIC and REP-PCR for VRE

	Standardized cycling parameters		
Steps	**ERIC-PCR**	**REP-PCR**	**No. of cycles**
Initial denaturation	95˚C for 5 min	95˚C for 5 min	1
Denaturation	94˚C for 1 min	94˚C for 45 sec	40
Annealing	25˚C for 1 min	40˚C for 1 min	
Extension	72˚C for 2 min	65˚C for 10 min	
Final extension	72˚C for 10 min	65˚C for 20 min	1
Hold/stand by	4˚C for 10 min	4˚C for 10 min	---

3.14.5 Discriminatory power of ERIC-PCR and REP-PCR genotyping techniques for VRE

Numerical index of discrimination was calculated by Simpson's index of diversity (Hunter and Gaston, 1988) using the formula –

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j (n_j - 1)$$

Where **D** = Simpson's index of diversity (Discriminatory power)

N = Total number of strains in the sample population

$\mathbf{n_j}$ = Number of strains belonging to the j^{th} type

s = Total number of types defined.

3.15 PRESERVATION OF ISOLATES

Following identification and confirmation, all the *Enterococci* isolates were picked up onto nutrient agar slants and stored at 4˚C as pure culture. Consecutive subculturing was done at regular intervals of 15-30 days.

CHAPTER IV

RESULTS

4.1 SAMPLING DETAILS

A total of 780 samples were collected including food samples from poultry (173 chicken and 61 quail); food samples of animal origin like (80 mutton, 56 raw pork, 54 fish, 52 cara beef, and 82 milk from bulk milk centres; 85 faecal swabs (32 chicken cloacal swabs, 18 buffalo rectal swabs, 23 sheep rectal swabs and 12 pig rectal swabs); 25 water samples; 40 uterine discharges of animals as well as 42 stool samples from veterinary students, 12 human diarrhoeic stool and 18 human urine samples which were collected from different places of Krishna district were processed for isolation of *Enterococcus* spp. and confirmed by molecular techniques like PCR. The phenotypic detection of virulence factors was done to evaluate pathogenic potential of *Enterococcus* isolates and two m-PCR assays targeting *gelE*, *ace*, *efaA*, *esp*, *asa*, *hyl* and *cylA* were used for detection of virulence genes in *Enterococcus* spp. The *Enterococcus* spp. were also characterized invitro for antibiotic resistance patterns (phenotypically and genotypically) and genetic diversity studies among VRE by using ERIC-PCR and REP-PCR.

4.2 ISOLATION AND IDENTIFICATION OF *ENTEROCOCCUS* SPECIES

4.2.1 Cultural isolation and phenotypic characterization of *Enterococcus* spp.

Isolation of *Enterococcus* spp. included 2 steps, enrichment in non-selective broth medium, BHI for 16-20h at 37^0C followed by plating on KF- streptococcal agar and KAA agar for 24 to 48h at 37^0C to enable the recognition of *Enterococcus* colonies. Enterococci isolates were selected based on specific colony characteristics viz., deep red colour colonies on KF-streptococcal agar (Fig-3) and transparent colonies surrounded

with black haloes on KAA agar (Fig-4). Out of 780 samples analyzed, 681 (87.30%) samples were found to be positive for *Enterococcus* spp. by cultural methods.

4.2.2 Confirmation of *Enterococcus* spp. by biochemical tests

All the *Enterococcus* isolates obtained from different sources by cultural methods were subjected to Gram's staining procedure and were found to be Gram positive rods (Fig-5). All 681 *Enterococcus* suspected isolates were confirmed by subjecting to biochemical tests such as oxidase test (Fig-6), hippurate hydrolysis (Fig-7), catalase test (Fig-8), Voges Proskaeur test (Fig-9) and Aesculin hydrolysis (Fig-10). Details of all biochemical reactions were mentioned in Table- 18.

Table-18: Different biochemical reactions of *Enterococcus* spp.

Test	Reaction
Gram staining	**Gram Positive**
Oxidase	**Negative**
Hippurate hydrolysis	**Positive**
Catalase	**Negative**
Voges-Proskauer test	**Positive**
Aesculin hydrolysis	**Positive**

4.3 MOLECULAR CONFIRMATION OF GENUS *ENTEROCOCCUS* BY PCR

All 681 *Enterococcus* isolates were subjected to *Enterococcus* genus 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. Out of 681 *Enterococcus* isolates that were positive by cultural and biochemical tests, 677 were confirmed genotypically by genus specific PCR (Fig-11). Distribution of *Enterococcus* spp. isolated from different sources are listed in Table-19.

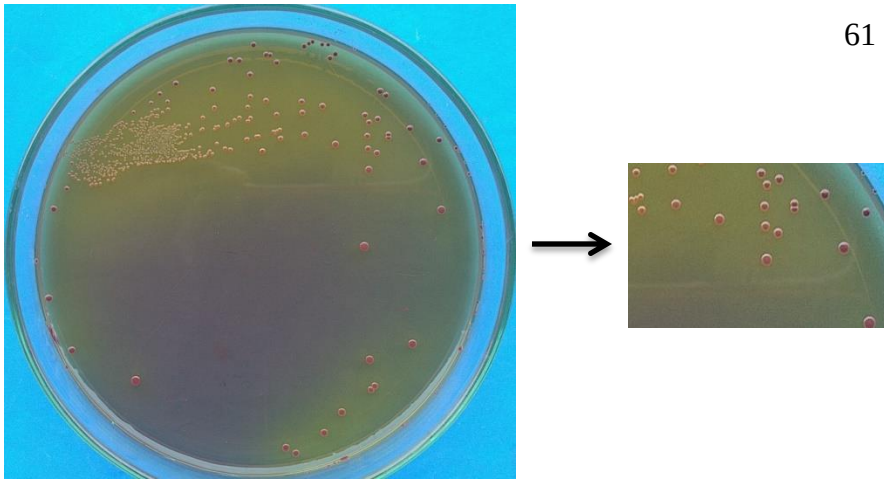


Fig-3: Deep red colonies of *Enterococcus* spp. on KF-streptococcal agar

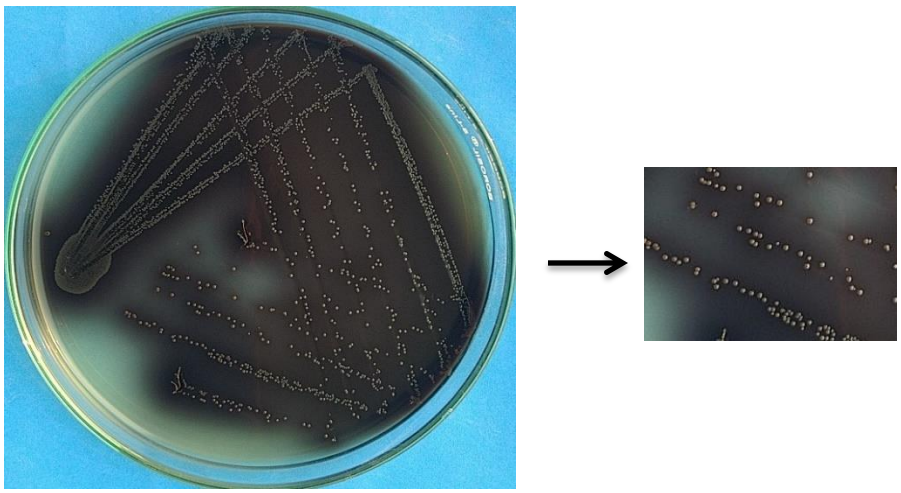


Fig-4: White to grey, trasparent and convex colonies of *Enterococcus* spp. on KAA agar

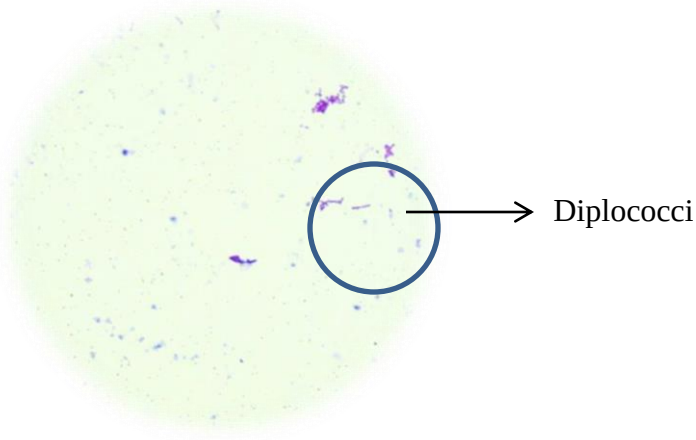


Fig-5: Grams staining image showing Gram positive rods of *Enterococcus* spp.

Fig-6: Oxidase test in *Enterococcus* species

A- *Enterococcus* spp. isolate (negative reaction)
B- Positive control (*Campylobacter coli* ATCC 33559) showing blue colour

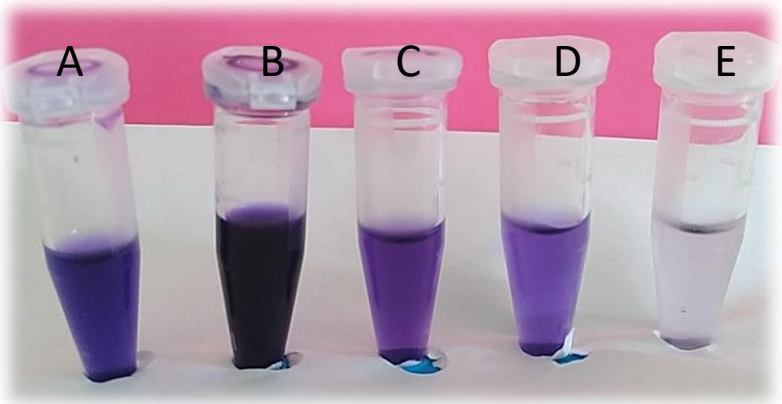


Fig-7: Hippurate hydrolysis in *Enterococcus* spp.

A- *E. faecalis* isolate showing positive reaction (blue colour)
B- *E. faecium* isolate showing positive reaction
C- *E. gallinarum* isolate showing positive reaction
D- *E. casseliflavus* isolate showing positive reaction
E- Negative control ((*E. coli* ATCC15243)

Fig-8: Catalase test in *Enterococcus* spp.

A- *E. faecalis* isolate negative for catalase test
B- Positive control (*E. coli* ATCC15243) showing elaboration of oxygen bubbles

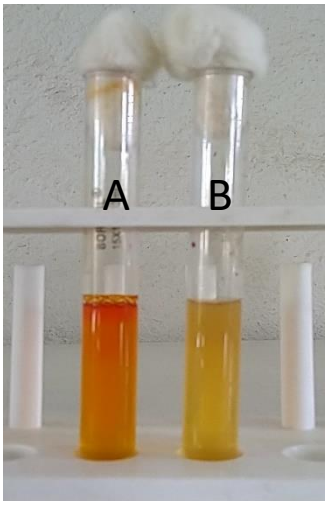


Fig-9: Voges Proskauer test in *Enterococcus* spp.

A- *E. faecalis* isolate showing poistive reaction (red colour)
B- Negative control (*E. coli* ATCC15243)

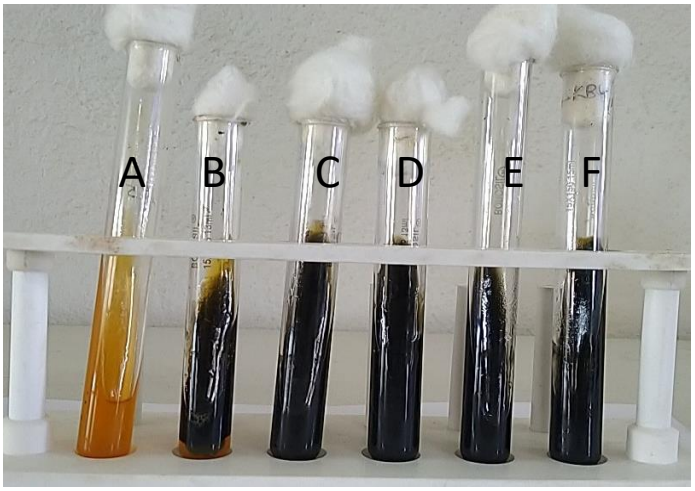


Fig-10: Aesculin hydrolysis in *Enterococcus* spp.

A- Negative control (*E. coli* ATCC15243)
B- *E. faecalis* showing positive reaction
C- *E. faecium* showing positive reaction
D- *E. gallinarum* showing positive reaction
E- *E. casseliflavus* showing positive reaction
F- Positive control (*E. faecalis* MTCC439)

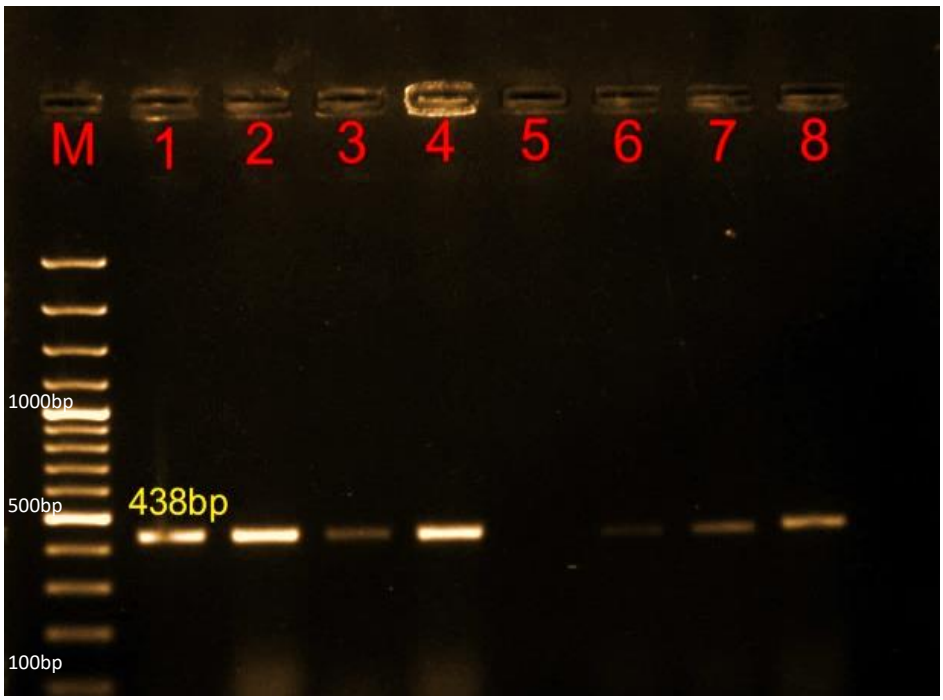


Fig-11: Gel photograph of PCR showing genus specific bands for *Enterococcus*

Lane M Molecular weight marker (100-3000bp)

Lane 1 Positive control of *E. faecalis* MTCC439 showing genus specific gene (partial sequence of *sodA*) (438 bp)

Lane 2 Positive control of *E. gallinarum* MTCC7049 showing genus specific gene (438bp)

Lane 3 Known positive standard of *E. faecium* showing genus specific gene (438 bp)

Lane 4 Known positive standard of *E. casseliflavus* showing genus specific gene (438 bp)

Lane 5 Negative control

Lane 6-8 Genus *Enterococcus* positive isolates from different samples showing genus specific gene (chicken, mutton and pork, respectively)

4.4 MOLECULAR DETECTION OF *ENTEROCOCCUS* SPP. BY USING m-PCR

All the 677 *Enterococcus* confirmed isolates were subjected to two *Enterococcus* species specific m-PCR assays 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. Out of 677 *Enterococcus* isolates, 278 *E. faecalis* (41.06%), 179 *E. faecium* (26.44%), 103 *E. gallinarum* (15.21%) and 48 *E. casseliflavus* (7.09%) were confirmed by m-PCR (Table-19 & 20 and Fig-12).

Table-19: Prevalence of *Enterococcus* spp. among different sources

Source	No. of samples examined	No. of samples positive for genus *Enterococcus* (%)	Isolates positive for species of *Enterococcus* spp (*E. faecalis, E. faecium, E. gallinarum* and *E. casseliflavus*)
FOOD SAMPLES OF ANIMAL ORIGIN			
Chicken	173	148 (85.54)	142 (95.94)
Quail	61	56 (91.80)	52 (92.85)
Mutton	80	61 (76.25)	44 (72.13)
Pork	56	54 (96.42)	46 (85.18)
Fish	54	47 (87.03)	47 (100)
Carabeef	52	49 (94.23)	45 (91.83)
Milk from bulk milk collection centers	82	73 (89.02)	69 (94.52)
TOTAL	**558**	**488 (87.45)**	**445 (91.18)**
ANIMAL FAECAL SWABS			
Chicken cloacal swabs	32	29 (90.62)	23 (79.31)
Sheep rectal swabs	23	23 (100)	23 (100)
Buffalo rectal swabs	18	17 (94.44)	16 (94.11)
Pig rectal swabs	12	12 (100)	11 (91.66)
TOTAL	**85**	**81 (95.29)**	**73 (90.12)**
HUMAN SAMPLES			
Human Stool samples	42	39 (92.85)	36 (92.30)
Human diarrhoeic stool samples	12	12 (100)	12 (100)
Human urine samples	18	10 (55.55)	6 (60.00)
TOTAL	**72**	**61 (84.72)**	**54 (88.52)**
ENVIRONMENTAL SAMPLES			
Water	**25**	**25 (100)**	**22 (88.00)**
MISCELLANEOUS			
Uterine discharges	40	22 (55.00)	14 (63.63)
Total	**780**	**677 (86.79)**	**608 (89.80)**

TABLE-20: *Enterococcus* species isolated from different samples by species specific m-PCR

Source	Total No. of *Enterococcus* isolates (%)	*E. faecalis* (%)	*E. faecium* (%)	*E. gallinarum* (%)	*E. casseliflavus* (%)
FOOD SAMPLES ANIMAL ORIGIN					
Chicken (173)	148 (85.54)	62 (41.89)	46 (31.08)	27 (18.24)	7 (4.72)
Quail (61)	56 (91.80)	21 (37.50)	15 (26.78)	8 (14.28)	8 (14.28)
Mutton (80)	61 (76.25)	26 (42.62)	2 (3.27)	6 (9.83)	10 (16.39)
Pork (56)	54 (96.42)	32 (59.25)	9 (16.66)	2 (3.70)	3 (5.55)
Fish (54)	47 (87.03)	27 (57.44)	4 (8.51)	14 (29.78)	2 (4.25)
Carabeef (52)	49 (94.23)	3 (6.12)	32 (65.30)	6 (12.24)	4 (8.16)
Milk from bulk milk collection centers (82)	73 (89.02)	28 (38.35)	22 (30.13)	11 (15.06)	8 (10.95)
TOTAL	**488 (87.45)**	**199 (40.77)**	**130 (26.63)**	**74 (15.16)**	**42 (8.60)**
ANIMAL FAECAL SWABS					
Chicken cloacal swabs (32)	29 (90.62)	10 (34.48)	4 (13.79)	7 (24.13)	2 (6.89)
Sheep rectal swabs (23)	23 (100)	14 (60.86)	7 (30.43)	0	2 (8.69)
Buffalo rectal swabs (18)	17 (94.44)	10 (58.82)	5 (29.41)	1 (5.88)	0
Pig rectal swabs (12)	12 (100)	5 (41.66)	5 (41.66)	1 (8.33)	0
TOTAL	**81 (95.29)**	**39 (48.14)**	**21 (25.92)**	**9 (11.11)**	**4 (4.93)**
HUMAN SAMPLES					
Human stool samples (42)	39 (92.85)	8 (20.51)	16 (41.02)	11 (28.20)	1 (2.56)
Human diarrhoeic stool samples (12)	12 (100)	5 (41.66)	4 (33.33)	3 (25.00)	0
Human urine samples (18)	10 (55.55)	6 (60.00)	0	0	0
TOTAL	**61 (84.72)**	**19 (31.14)**	**20 (32.78)**	**14 (22.95)**	**1 (1.63)**
ENVIRONMENTAL SAMPLES					
Water (25)	25 (100)	12 (48.00)	5 (20.00)	4 (16.00)	1 (4.00)
MISCELLANEOUS					
Uterine discharges of animals (40)	22 (55.00)	9 (40.90)	3 (13.63)	2 (9.09)	0
TOTAL (780)	**677 (86.79)**	**278 (41.06)**	**179 (26.44)**	**103 (15.21)**	**48 (7.09)**

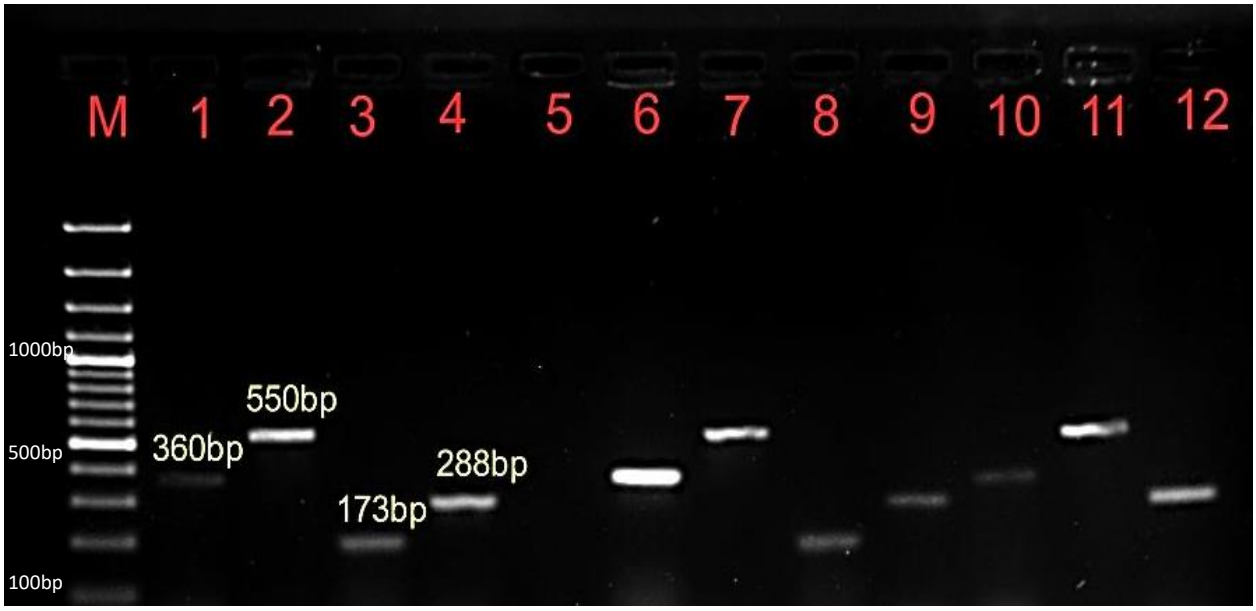


Fig-12: Gel photograph of PCR showing species specific bands for different *Enterococcus* spp. (*E. faecalis, E. faecium, E. gallinarum and E. casseliflavus*)

Lane M Molecular weight marker (100-3000bp)

Lane 1 Positive control of *E. faecalis* MTCC439 (360bp)

Lane 2 Known positive standard of *E. faecium* (550bp)

Lane 3 Positive control of *E. gallinarum* MTCC7049 (173bp)

Lane 4 Known positive standard of *E. casseliflavus* (288bp)

Lane 5 Negative control

Lane 6 *E. faecalis* positive isolate from chicken sample (360bp)

Lane 7 *E. faecium* positive isolate from pork sample (550bp)

Lane 8 *E. gallinarum* positive isolate from mutton sample (173bp)

Lane 9 *E. casseliflavus* positive isolate from human stool sample (288bp)

Lane 10 *E. faecalis* positive isolate from uterine discharge (360bp)

Lane 11 *E. faecium* positive isolate from water sample (550bp)

Lane 12 *E. casseliflavus* positive isolate from quail sample (288bp)

4.5 PHENOTYPIC DETECTION OF VIRULENCE FACTORS IN *ENTEROCOCCUS* SPP.

A total of 608 *Enterococcus* isolates of different species from different sources were subjected for detection of virulence factors by phenotypic methods like slime layer formation (Fig-13), gelatinase (Fig-14), DNase (Fig-15), caseinase activity (Fig-16), lipase activity (Fig-17), haemolytic activity on sheep RBC (Fig-18), biofilm formation (Fig-19 and 20) and HA test (Fig-21). The phenotypic virulence factors among 608 *Enterococcus* isolates i.e. hemolysis activity of sheep RBC, slime layer, lipase activity, caseinase activity, biofilm formation, gelatinase, DNase activity and HA test were detected in 312 (51.31%), 243 (39.96%), 47 (7.73%), 121 (19.90%), 236 (38.81%), 141 (23.19%), 37 (6.08%) and 87 (14.30%) *Enterococcus* isolates, respectively (Table-21 and 22 and Fig-22).

Table-21: Distribution of phenotypic virulence factors in different *Enterococcus* spp.

Phenotypic pattern	*E. faecalis* (278)	*E. faecium* (179)	*E. gallinarum* (103)	*E. casseliflavus* (48)	Total (608)
Hemolysis of sheep RBC	146 (52.21%)	79 (44.13%)	71 (68.93%)	16 (33.33%)	**312 (51.31%)**
Slime layer	155 (55.75%)	48 (26.81%)	9 (8.73%)	31 (64.58%)	**243 (39.96%)**
Lipase activity	34 (12.23%)	9 (5.02%)	0	4 (8.33%)	**47 (7.73%)**
Caseinase activity	64 (23.02%)	25 (13.96%)	24 (23.30%)	8 (16.66%)	**121 (19.90%)**
Biofilm formation	111 (39.92%)	67 (37.43%)	41 (39.80%)	17 (35.41%)	**236 (38.81%)**
Gelatinase	67 (31.29%)	44 (24.58%)	21 (20.38%)	9 (18.75%)	**141 (23.19%)**
DNase activity	14 (5.03%)	20 (11.17%)	3 (2.91%)	0	**37 (6.08%)**
HA test	37 (13.30%)	41 (22.90%)	9 (8.73%)	0	**87 (14.30%)**

TABLE-22: Distribution of phenotypic virulence factors in different *Enterococcus* spp. isolated from different sources

Samples (No. of *Enterococcus* isolates)	Hemolysis of sheep RBC (%)	Slime layer (%)	Lipase activity (%)	Caseinase activity (%)	Biofilm formation (%)	Gelatinase (%)	DNase activity (%)	HA test (%)
FOODS OF ANIMAL ORIGIN								
Chicken (142)	71 (50.00)	61 (42.95)	0	10 (7.04)	49 (34.50)	17 (11.97)	3 (2.11)	4 (2.81)
Quail (52)	19 (36.53)	23 (44.23)	4 (7.69)	4 (7.69)	18 (34.61)	15 (28.84)	0	2 (3.84)
Mutton (44)	27 (61.36)	19 (43.18)	6 (13.63)	6 (13.63)	17 (38.63)	16 (36.36)	0	11 (25.00)
Pork (46)	23 (50.00)	12 (26.08)	2 (4.34)	2 (4.34)	14 (30.43)	0	14 (30.43)	0
Fish (47)	34 (72.34)	22 (46.80)	8 (17.02)	17 (36.17)	20 (42.55)	12 (25.53)	0	0
Carabeef (45)	0	7 (15.55)	4 (8.88)	16 (35.55)	19 (42.22)	0	0	0
Milk from bulk milk collection centers (69)	39 (56.52)	9 (13.04)	7 (10.14)	6 (8.69)	11 (15.94)	17 (24.63)	0	0
TOTAL (445)	**213 (47.86)**	**153 (34.38)**	**31 (6.96)**	**61 (13.70)**	**148 (33.25)**	**77 (17.30)**	**17 (3.82)**	**17 (3.82)**
ANIMAL FAECAL SWABS								
Chicken cloacal swabs (23)	13 (56.52)	14 (60.86)	4 (17.39)	4 (17.39)	12 (52.17)	11 (47.82)	12 (52.17)	14 (60.86)
Sheep rectal swabs (23)	12 (52.17)	3 (13.04)	0	10 (43.47)	8 (34.78)	9 (39.13)	0	5 (21.73)
Buffalo rectal swabs (16)	7 (43.75)	14 (87.50)	0	7 (43.75)	5 (31.25)	6 (37.5)	0	0
Pig rectal swabs (11)	1 (9.09)	3 (27.27)	0	3 (27.27)	7 (63.63)	5 (45.45)	0	0
TOTAL	**33 (45.20)**	**34 (46.57)**	**4 (5.47)**	**24 (33.87)**	**32 (43.83)**	**31 (42.46)**	**12 (16.43)**	**19 (26.02)**
HUMAN SAMPLES								
Human stool samples (36)	27 (75.00)	13 (36.11)	6 (16.66)	6 (16.66)	14 (38.88)	0	0	17 (47.22)
Human diarrhoeic stool samples (12)	0	7 (58.33)	2 (16.66)	2 (16.66)	4 (33.33)	7 (58.33)	3 (25.00)	3 (25.00)
Human urine samples (6)	6 (100)	6 (100)	0	5 (83.33)	5 (83.33)	6 (100)	5 (83.33)	0
TOTAL	**33 (61.11)**	**26 (48.14)**	**8 (14.81)**	**13 (24.07)**	**23 (42.59)**	**13 (24.07)**	**8 (14.81)**	**20 (37.03)**
ENVIRONMENTAL SAMPLES								
Water (22)	21 (95.45)	16 (72.72)	3 (13.63)	10 (45.45)	22 (100)	9 (40.90)	0	19 (86.36)
MISCELLANEOUS								
Uterine discharges (14)	12 (85.71)	14 (100)	1 (7.14)	13 (92.85)	11 (78.57)	11 (78.57)	0	12 (85.71)
Total (608)	**312 (51.31)**	**243 (39.96)**	**47 (7.73)**	**121 (19.90)**	**236 (38.81)**	**141 (23.19)**	**37 (6.08)**	**87 (14.30)**

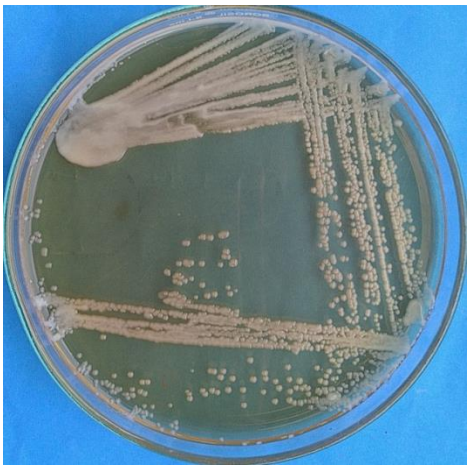


Fig-13: Slime layer formation of *Enterococcus* spp. on BHI agar

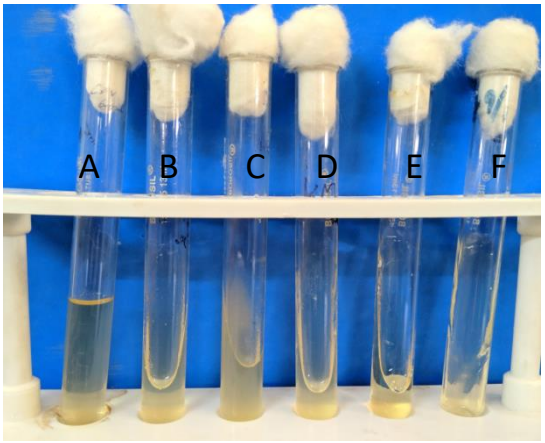


Fig-14: Gelatinase activity of *Enterococcus* spp.

A : Positive control of *Staphylococcus aureus* known standard isolate
B-F : Variable gelatinase activity of different *Enterococcus* isolates

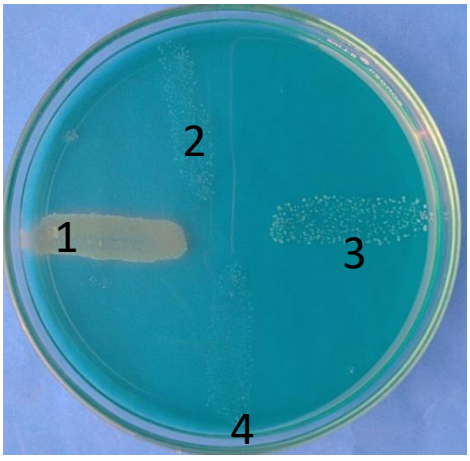


Fig-15: DNase activity of *Enterococcus* spp.

1 : *Enterococcus* isolate showing positive reaction (Yellow colour)
2-4: *Enterococcus* isolates showing negative reaction

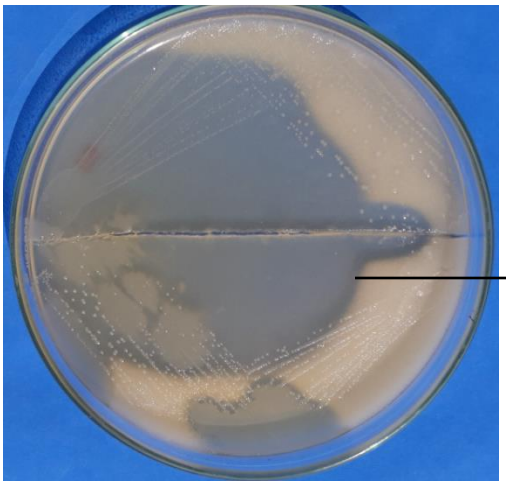


Fig-16: Caseinase activity of *Enterococcus* spp. on skim milk agar

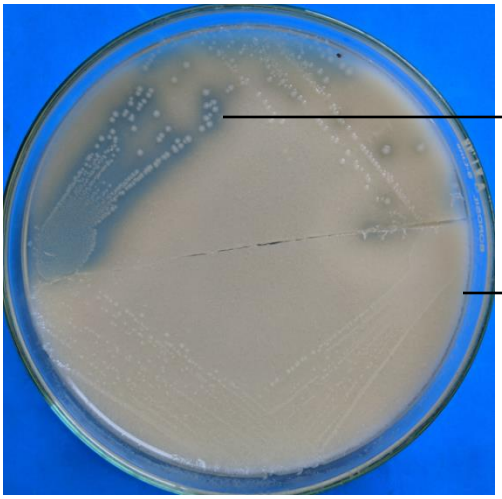


Fig-17: Lipolysis activity of *Enterococcus* spp. on egg yolk agar

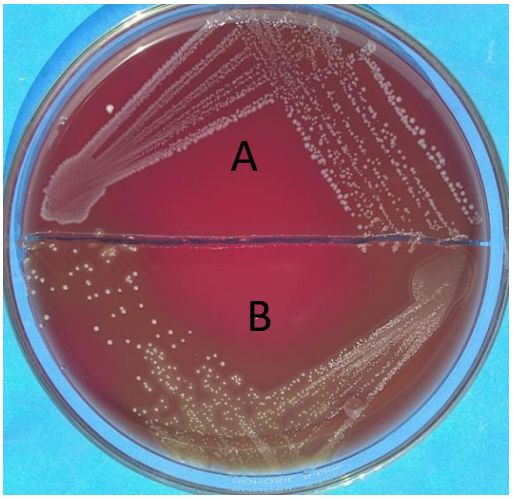


Fig-18: Hemolysis of *Enterococcus* spp. on blood agar plate

A- No hemolysis
B- Hemolysis observed around the colonies

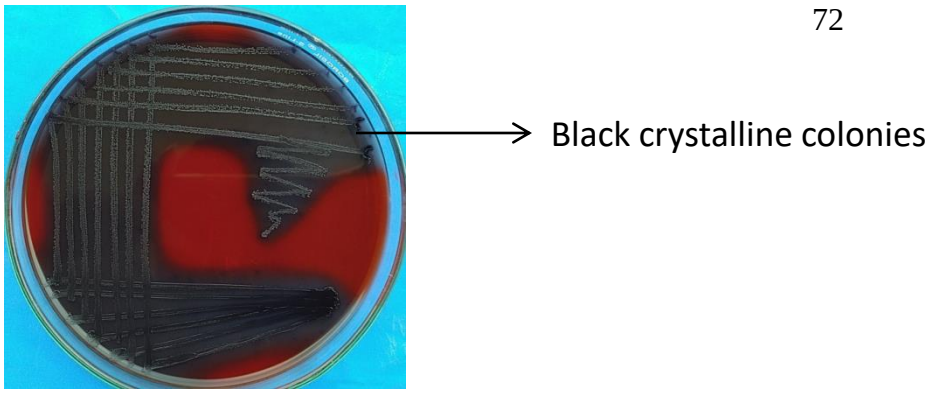


Fig-19: Biofilm formation of *Enterococcus* spp. on Congo red agar

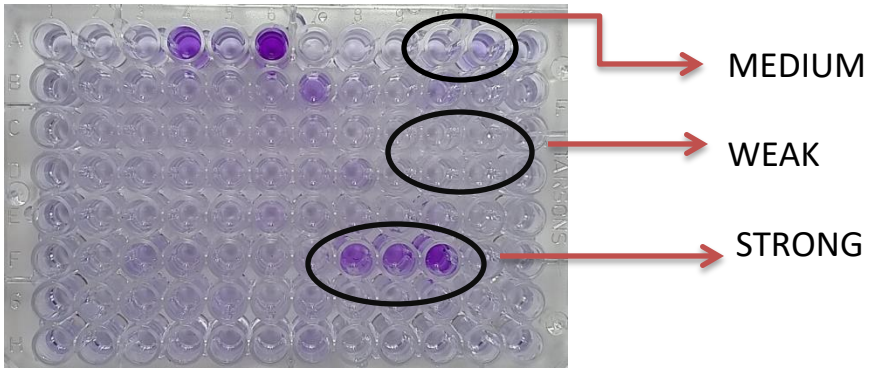


Fig-20: Strength of biofilm formation in Enterococcus spp. detected by MTP assay

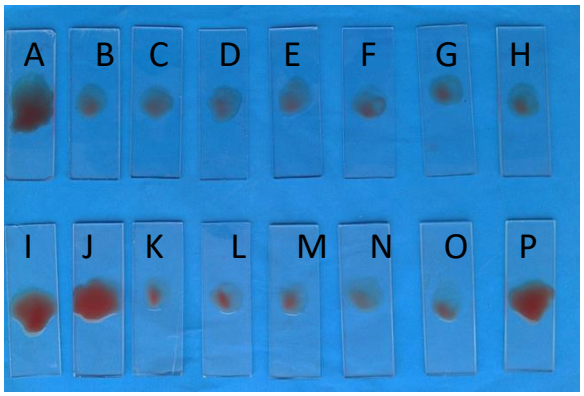


Fig-21: HA test of *Enterococcus* spp. on 5% sheep RBC

A-P: Variable hemaglutination of sheep RBC by differnet *Enterococcus* isolates

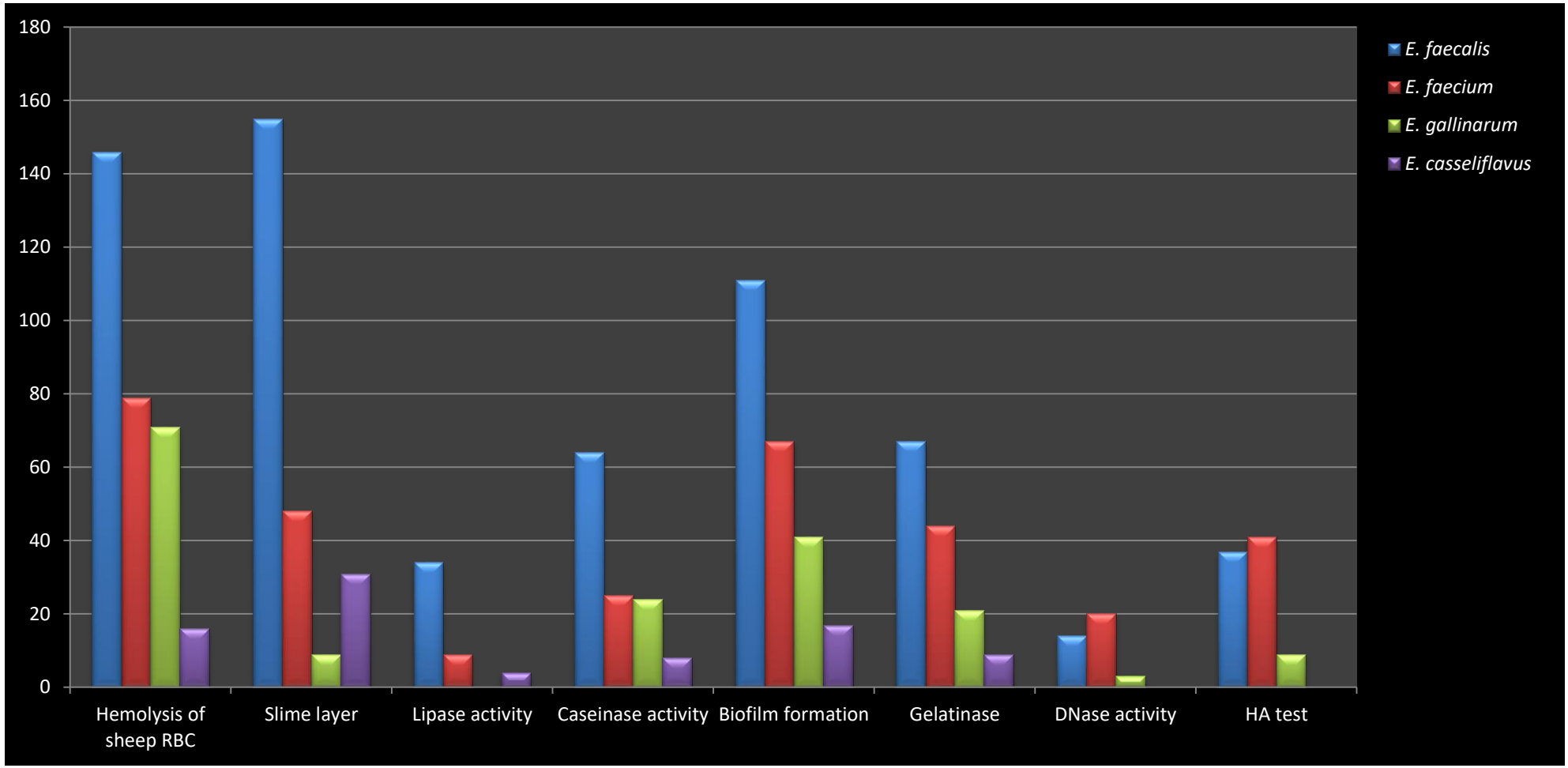


Fig-22: Bar diagram showing distribution of virulence factors in different *Enterococcus* spp. by phenotypic method

4.6 DETECTION OF VIRULENCE GENES IN *ENTEROCOCCUS* SPP. BY m-PCR

A total of 608 *Enterococcus* isolates of different species from different sources were subjected to two m-PCR assays for detection of virulence genes like *gelE*, *ace*, *efaA*, *esp*, *asa*, *hyl* and *cylA* (Fig-23 and Fig-24). The genes *gelE*, *ace*, *efaA*, *asa*, *hyl* and *cylA* were detected in 181 (29.76%), 117 (19.24%), 101 (16.61%), 180 (29.60%), 131 (21.54%) and 68 (11.11%) *Enterococcus* isolates, respectively (Table-23 and 24). None of the isolates showed *esp* gene.

Table-23: Virulence gene profile of different *Enterococcus* spp.

Species	*gelE* (%)	*ace* (%)	*efaA* (%)	*asa* (%)	*hyl* (%)	*cylA* (%)
***E. faecalis* (278)**	85 (30.57)	61 (21.94)	49 (17.62)	70 (25.17)	56 (20.14)	31 (11.15)
***E. faecium* (179)**	45 (25.13)	30 (16.75)	23 (12.84)	56 (31.28)	40 (22.34)	20 (11.17)
***E. gallinarum* (103)**	33 (32.03)	17 (16.50)	18 (17.47)	33 (32.03)	24 (23.30)	12 (11.65)
***E. cassiliflavus* (48)**	18 (37.50)	9 (18.75)	11 (22.91)	21 (43.75)	11 (22.91)	5 (10.41)
Total (608)	**181 (29.76)**	**117 (19.24)**	**101 (16.61)**	**180 (29.60)**	**131 (21.54)**	**68 (11.18)**

TABLE-24: Virulence gene profile of *Enterococcus* spp. isolated from different sources

Samples (No. of *Enterococcus* isolates)	*gelE* (%)	*ace* (%)	*efaA* (%)	*asa* (%)	*hyl* (%)	*cylA* (%)
FOOD SAMPLES ANIMAL ORIGIN						
Chicken (142)	45 (31.69)	31 (21.83)	28 (19.71)	42 (29.57)	31 (21.83)	18 (12.67)
Quail (52)	10 (19.23)	7 (13.46)	7 (13.46)	16 (30.76)	8 (15.38)	1 (1.92)
Mutton (44)	17 (38.63)	9 (20.45)	4 (9.09)	19 (43.18)	12 (27.27)	4 (9.09)
Pork (46)	11 (23.91)	9 (19.56)	7 (15.21)	8 (17.39)	7 (15.21)	6 (13.04)
Fish (47)	10 (21.27)	6 (12.76)	5 (10.63)	20 (42.55)	16 (34.04)	6 (12.76)
Carabeef (45)	5 (11.11)	0	0	4 (8.88)	1 (2.22)	0
Milk from bulk milk collection centers (69)	9 (13.04)	6 (8.69)	6 (8.69)	5 (7.24)	3 (4.34)	5 (7.24)
TOTAL	**107 (24.04)**	**68 (15.28)**	**51 (11.46)**	**114 (25.61)**	**78 (17.52)**	**40 (8.98)**
ANIMAL FAECAL SWABS						
Chicken cloacal swabs (23)	11 (47.82)	3 (13.04)	2 (8.69)	8 (34.78)	1 (4.34)	0
Sheep rectal swabs (23)	12 (52.17)	7 (30.43)	4 (17.39)	13 (56.52)	10 (43.47)	8 (34.78)
Buffalo rectal swabs (16)	6 (37.50)	9 (56.25)	8 (50.00)	5 (31.25)	6 (37.50)	0
Pig rectal swabs (11)	2 (18.18)	4 (36.36)	3 (27.27)	2 (18.18)	4 (36.36)	6 (54.54)
TOTAL	**31 (42.46)**	**23 (31.50)**	**17 (23.28)**	**28 (38.35)**	**21 (28.76)**	**14 (19.17)**
HUMAN SAMPLES						
Human stool samples (36)	15 (41.66)	8 (22.22)	6 (16.66)	13 (36.11)	6 (16.66)	2 (5.55)
Human diarrhoeic stool samples (12)	4 (33.33)	2 (16.66)	4 (33.33)	2 (16.66)	3 (25.00)	0
Human urine samples (6)	3 (50.00)	2 (33.33)	2 (33.33)	2 (33.33)	4 (66.66)	2 (33.33)
TOTAL	**22 (40.74)**	**12 (22.22)**	**12 (22.22)**	**17 (31.48)**	**13 (24.07)**	**4 (7.40)**
ENVIRONMENTAL SAMPLES						
Water (22)	15 (68.18)	10 (45.45)	12 (54.54)	12 (54.54)	13 (59.090	5 (22.72)
MISCELLANEOUS						
Uterine discharges (14)	6 (42.85)	4 (28.57)	3 (21.42)	9 (64.28)	6 (42.85)	5 (35.71)
Total (608)	**181 (29.76)**	**117 (19.24)**	**101 (16.61)**	**180 (29.60)**	**131 (21.54)**	**68 (11.18)**

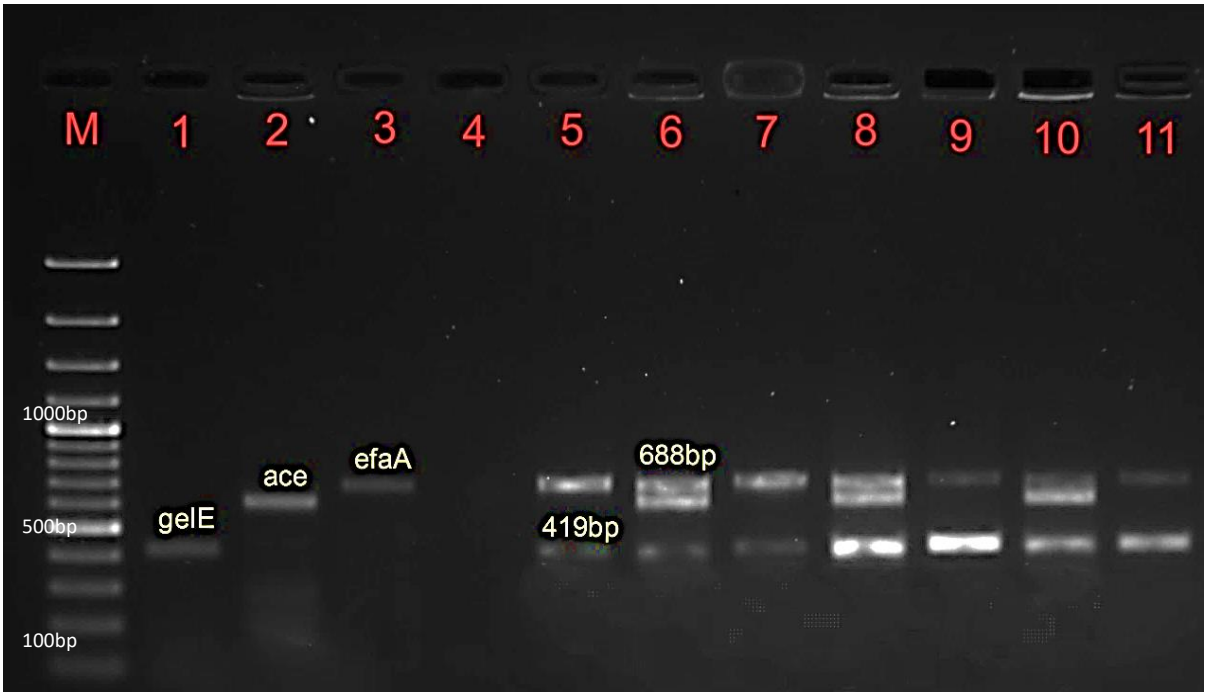


Fig-23: m-PCR-I showing *gelE*, *ace* and *efaA* virulence genes in *Enterococcus* spp.

Lane M Molecular weight marker (100-3000bp)

Lane 1 Positive control of *gelE* gene in *E. faecalis* MTCC439 (419bp)

Lane 2 Positive control of *ace* gene in *E. faecalis* MTCC439 (616bp)

Lane 3 Positive control of *efaA* gene in *E. faecalis* MTCC439 (688bp)

Lane 4 Negative control

Lane 5 *E. faecalis* positive isolate showing *gelE* and *efaA* genes

Lane 6 *E. faecalis* positive isolate showing *gelE, ace* and *efaA* genes

Lane 7 *E. faecium* positive isolate showing *gelE* and *efaA* genes

Lane 8 *E. faecium* positive isolate showing *gelE, ace* and *efaA* genes

Lane 9 *E. gallinarum* positive isolate showing *gelE* and *efaA* genes

Lane 10 *E. gallinarum* positive isolate showing *gelE, ace* and *efaA* genes

Lane 11 *E. casseliflavus* positive isolate showing *gelE* and *efaA* genes

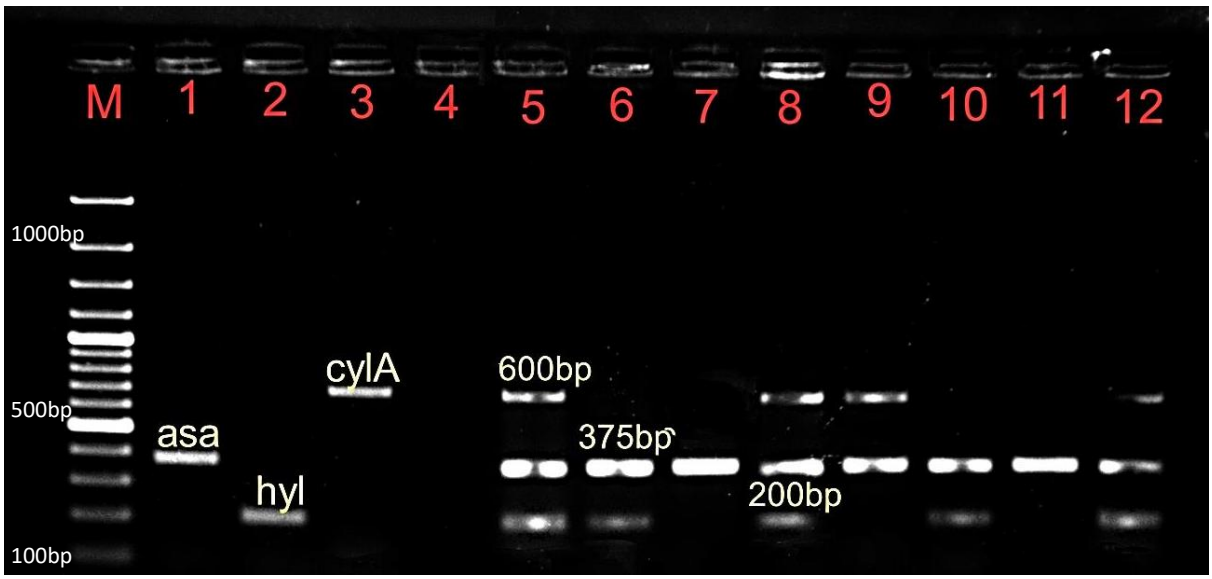


Fig-24: m-PCR-II showing *asa*, *hyl* and *cylA* virulence genes in *Enterococcus* spp.

Lane M Molecular weight marker (100-3000bp)

Lane 1 Positive control of *asa* gene in *E. faecalis* MTCC439 (375 bp)

Lane 2 Positive control of *hyl* gene in *E. faecalis* MTCC439 (200 bp)

Lane 3 Positive control of *cylA* gene in *E. gallinarum* MTCC7049 (600 bp)

Lane 4 Negative control

Lane 5 *E. faecalis* positive isolate showing *asa*, *hyl* and *cylA* genes

Lane 6 *E. faecalis* positive isolate showing *asa* and *hyl* genes

Lane 7 *E. faecium* positive isolate showing *asa* gene

Lane 8 *E. faecium* positive isolate showing *asa*, *hyl* and *cylA*

Lane 9 *E. gallinarum* positive isolate showing *asa* and *cylA* genes

Lane 10 *E. gallinarum* positive isolate showing *asa* and *hyl* genes

Lane 11 *E. casseliflavus* positive isolate showing *asa* gene

Lane 12 *E. casseliflavus* positive isolate showing *asa*, *hyl* and *cylA* genes

4.6.1 Phenotypic and genotypic correlation of biofilm formation in *Enterococcus* isolates

A total of 608 isolates of different species of enterococci were subjected to preliminary identification of biofilm formation on Congo red agar. Out of 608 *Enterococcus* isolates, 236 isolates showed black colonies with crystalline consistency indicating biofilm formation. The strength of biofilm formation was scored by MTP assay. Phenotypic (gelatinase) and genotypic (*gelE*, *ace*, *efaA* and *asa*) correlation of biofilm formation in *Enterococcus* isolates was listed in Table-25. Correlation between phenotypic virulence factors (gelatinase, slime layer and hemolysin) and virulence genes (*gelE*, *asa* and *cylA*) in *Enterococcus* spp. were mentioned in Table-26.

Table-25: Phenotypic and genotypic correlation of biofilm formation in *Enterococcus* isolates

Isolate ID	Species	Phenotype		Genotype			
		Biofilm strength	Gelatinase	*gelE*	*ace*	*efaA*	*asa*
q44	*E. cassiliflavus*	+	+	+	+	+	+
c141	*E. cassiliflavus*	+++	-	+	+	+	+
c119	*E. cassiliflavus*	+++	+	+	+	+	+
c13	*E. cassiliflavus*	++	+	+	+	+	+
w10	*E. cassiliflavus*	+++	+	+	+	+	+
c142	*E. cassiliflavus*	+	+	+	-	+	+
c93	*E. cassiliflavus*	+	+	-	+	+	+
m30	*E. cassiliflavus*	+++	-	+	-	+	+
c122	*E. cassiliflavus*	++	+	+	+	+	-
m87	*E. cassiliflavus*	++	+	+	-	-	+
CC16	*E. cassiliflavus*	++	-	+	-	-	+
c32	*E. cassiliflavus*	+	-	+	-	-	+
c66	*E. cassiliflavus*	+	-	+	-	-	+
f35	*E. cassiliflavus*	+	-	+	-	-	+
p17	*E. cassiliflavus*	+	-	+	-	-	-
p55	*E. cassiliflavus*	++	-	+	-	+	-
h19	*E. cassiliflavus*	+	+	+	-	-	+
c39	*E. faecalis*	+++	+	+	+	+	+
c110	*E. faecalis*	+	+	+	+	+	+
f46	*E. faecalis*	+++	+	+	+	+	+
m16	*E. faecalis*	++	+	+	+	+	+
p25	*E. faecalis*	++	+	+	+	+	+
w1	*E. faecalis*	++	+	+	+	+	+
w3	*E. faecalis*	+++	+	+	+	+	+
w17	*E. faecalis*	+	+	+	+	+	+

w24	*E. faecalis*	++	+	+	+	+	+
UD22	*E. faecalis*	+++	+	+	+	+	+
u4	*E. faecalis*	+	+	+	+	+	+
h24	*E. faecalis*	+++	+	+	+	+	+
q1	*E. faecalis*	++	+	+	+	+	+
c86	*E. faecalis*	+	+	+	+	+	+
c136	*E. faecalis*	+++	+	+	+	+	+
f26	*E. faecalis*	++	+	+	+	+	-
m12	*E. faecalis*	+++	+	+	+	+	+
m13	*E. faecalis*	+++	+	+	+	+	+
q14	*E. faecalis*	+	+	-	+	+	+
q51	*E. faecalis*	++	+	+	+	+	-
c1	*E. faecalis*	++	+	-	+	+	+
c17	*E. faecalis*	+	+	-	+	+	+
c21	*E. faecalis*	+++	+	+	+	-	+
c58	*E. faecalis*	+	-	-	+	+	+
c98	*E. faecalis*	++	+	-	+	+	+
c151	*E. faecalis*	++	-	+	+	+	-
CC21	*E. faecalis*	++	+	+	+	+	-
f2	*E. faecalis*	+	-	-	+	+	-
f23	*E. faecalis*	++	+	+	-	-	+
m17	*E. faecalis*	++	+	+	-	+	-
m28	*E. faecalis*	++	+	+	+	-	+
p11	*E. faecalis*	++	-	+	+	+	-
w9	*E. faecalis*	+++	+	+	+	+	-
h4	*E. faecalis*	+	+	+	+	+	+
h5	*E. faecalis*	+	-	+	-	+	+
u10	*E. faecalis*	+++	+	+	+	+	-
hd2	*E. faecalis*	++	+	+	+	-	+
c24	*E. faecalis*	+	+	+	-	-	+
c28	*E. faecalis*	+++	+	+	+	+	-
c106	*E. faecalis*	+	+	+	-	-	-
c108	*E. faecalis*	++	-	+	+	-	-
c114	*E. faecalis*	+	+	+	-	-	+
f15	*E. faecalis*	++	+	-	-	-	+
m19	*E. faecalis*	++	+	+	+	-	-
m27	*E. faecalis*	+	+	+	-	+	-
m73	*E. faecalis*	++	-	-	+	-	+
M1	*E. faecalis*	++	+	+	-	+	-
p27	*E. faecalis*	+	-	+	-	-	+
h10	*E. faecalis*	+++	+	+	+	+	-
UD13	*E. faecalis*	++	-	+	-	-	+
hd3	*E. faecalis*	++	+	+	-	-	+
hd6	*E. faecalis*	++	+	+	+	+	-
q10	*E. faecalis*	++	+	+	-	-	+
q32	*E. faecalis*	++	+	+	-	-	+
q35	*E. faecalis*	+	+	-	-	-	+
q37	*E. faecalis*	++	+	+	-	-	+
q41	*E. faecalis*	++	+	-	+	+	-
q43	*E. faecalis*	+++	+	+	-	-	+

q45	*E. faecalis*	++	-	+	-	-	+
c73	*E. faecalis*	+	+	+	-	-	+
c91	*E. faecalis*	+	-	+	-	-	+
c107	*E. faecalis*	++	+	+	+	-	-
c118	*E. faecalis*	+	+	+	-	-	-
c137	*E. faecalis*	+++	+	+	-	+	-
f11	*E. faecalis*	+	+	-	+	-	+
f22	*E. faecalis*	++	-	-	-	-	+
f33	*E. faecalis*	++	+	+	-	-	-
f51	*E. faecalis*	++	-	-	-	-	+
f53	*E. faecalis*	++	+	+	-	-	+
m2	*E. faecalis*	+	+	-	+	-	+
m9	*E. faecalis*	++	+	-	+	-	+
m34	*E. faecalis*	++	-	-	+	-	+
m41	*E. faecalis*	++	+	-	+	-	+
m47	*E. faecalis*	++	+	-	+	-	+
m75	*E. faecalis*	++	-	+	-	-	+
SR12	*E. faecalis*	+++	+	+	+	-	-
BR5	*E. faecalis*	+	+	+	-	+	-
BR6	*E. faecalis*	++	+	+	-	-	+
CC12	*E. faecalis*	+	+	+	-	-	+
CC19	*E. faecalis*	++	+	+	+	-	-
SR26	*E. faecalis*	++	+	+	+	-	-
SR24	*E. faecalis*	++	+	+	-	+	-
CC15	*E. faecalis*	++	+	+	+	-	-
M50	*E. faecalis*	++	-	+	-	-	-
M71	*E. faecalis*	++	+	-	-	+	-
p6	*E. faecalis*	+	+	+	+	-	-
w2	*E. faecalis*	++	+	+	-	+	-
w11	*E. faecalis*	+	+	+	-	-	+
h11	*E. faecalis*	++	+	+	-	+	-
UD12	*E. faecalis*	++	-	-	-	-	-
UD18	*E. faecalis*	++	+	+	-	-	+
u9	*E. faecalis*	++	+	+	-	-	+
h29	*E. faecalis*	++	-	+	-	+	-
h30	*E. faecalis*	++	+	+	-	-	+
c19	*E. faecalis*	+	+	+	-	-	-
c20	*E. faecalis*	++	-	-	+	-	-
c72	*E. faecalis*	++	+	-	-	-	+
c87	*E. faecalis*	++	-	-	-	-	+
c104	*E. faecalis*	+	-	-	-	-	-
f6	*E. faecalis*	++	+	-	-	-	+
m22	*E. faecalis*	+	+	-	-	+	-
m29	*E. faecalis*	+	+	-	+	-	-
BR9	*E. faecalis*	+++	+	+	-	-	-
SR22	*E. faecalis*	+++	+	+	-	-	-
cb39	*E. faecalis*	+	+	-	-	-	+
w20	*E. faecalis*	++	-	-	-	-	+
UD16	*E. faecalis*	++	-	-	-	-	+
h1	*E. faecalis*	++	+	-	+	-	-

h33	*E. faecalis*	++	+	+	-	-	-
h39	*E. faecalis*	++	-	-	-	-	+
h41	*E. faecalis*	+	+	+	-	-	-
c76	*E. faecium*	+++	+	+	+	+	+
c84	*E. faecium*	+++	-	+	+	+	+
CC6	*E. faecium*	+	-	+	+	+	+
UD23	*E. faecium*	+++	+	+	+	+	+
c47	*E. faecium*	+++	+	+	+	+	+
f41	*E. faecium*	+++	+	+	+	+	+
m10	*E. faecium*	+	+	+	+	-	+
m42	*E. faecium*	+	-	+	+	-	+
M45	*E. faecium*	+++	+	+	+	+	+
p14	*E. faecium*	+++	+	+	+	+	+
w6	*E. faecium*	+	-	+	-	+	+
h25	*E. faecium*	+	+	+	+	+	+
q11	*E. faecium*	+	-	-	+	+	+
c62	*E. faecium*	+	+	-	+	+	+
c81	*E. faecium*	+	+	-	+	+	-
c111	*E. faecium*	+	+	+	+	+	-
c154	*E. faecium*	+	-	+	-	-	+
f14	*E. faecium*	+++	-	+	+	+	+
m51	*E. faecium*	+	-	-	+	+	+
p32	*E. faecium*	+++	+	+	+	+	+
w8	*E. faecium*	+	+	+	+	-	+
UD19	*E. faecium*	+	+	+	+	-	+
q13	*E. faecium*	+	-	-	+	+	+
q24	*E. faecium*	+	-	+	-	-	+
c59	*E. faecium*	+	+	-	+	+	+
c64	*E. faecium*	+	+	+	-	-	+
m3	*E. faecium*	+	+	+	-	-	-
m35	*E. faecium*	+	+	+	-	-	-
m48	*E. faecium*	+	-	+	-	-	-
M36	*E. faecium*	+	-	+	-	-	+
p5	*E. faecium*	+	+	+	-	-	+
p16	*E. faecium*	+	-	-	+	-	+
p54	*E. faecium*	+	+	+	+	+	-
w12	*E. faecium*	+	+	+	-	-	+
w21	*E. faecium*	+++	+	+	+	+	-
c15	*E. faecium*	+	+	+	+	-	-
c57	*E. faecium*	+	+	+	-	-	+
c71	*E. faecium*	+	+	-	-	-	+
c74	*E. faecium*	+++	+	-	+	+	-
c75	*E. faecium*	+	+	+	-	-	+
c80	*E. faecium*	+	+	+	-	-	+
c144	*E. faecium*	+	+	+	-	-	-
c156	*E. faecium*	+	+	-	-	-	+
f18	*E. faecium*	+	-	-	-	-	+
f30	*E. faecium*	+	-	-	-	-	+
f39	*E. faecium*	+	-	-	-	-	+
f40	*E. faecium*	+	+	-	-	-	+

m1	*E. faecium*	+	+	-	-	-	+
m31	*E. faecium*	+	-	-	-	-	+
SR2	*E. faecium*	+++	+	+	-	-	+
BR7	*E. faecium*	+	+	+	-	-	+
cb1	*E. faecium*	+	+	+	-	-	+
cb4	*E. faecium*	+	+	+	-	-	+
cb24	*E. faecium*	+	+	+	-	-	+
w22	*E. faecium*	+++	+	+	-	+	-
UD9	*E. faecium*	+	-	-	-	-	+
h31	*E. faecium*	+	-	+	-	-	+
q6	*E. faecium*	++	+	-	-	-	+
q8	*E. faecium*	+	-	-	-	-	+
c55	*E. faecium*	+	+	-	-	-	+
f1	*E. faecium*	+	+	-	-	-	+
f17	*E. faecium*	++	+	-	-	-	+
m11	*E. faecium*	+	+	+	-	-	-
m44	*E. faecium*	+	-	+	-	-	-
m53	*E. faecium*	+	-	-	+	-	-
SR9	*E. faecium*	++	+	+	-	-	-
SR23	*E. faecium*	++	-	-	-	-	+
c33	*E. gallinarum*	+++	+	+	+	+	+
c99	*E. gallinarum*	+++	-	+	+	+	+
h20	*E. gallinarum*	+	-	+	+	+	+
c31	*E. gallinarum*	+	-	+	+	+	+
h17	*E. gallinarum*	+	-	+	+	+	+
c79	*E. gallinarum*	+++	-	+	+	+	-
c112	*E. gallinarum*	+	-	+	+	+	-
c120	*E. gallinarum*	+	-	+	-	-	+
c159	*E. gallinarum*	+	-	+	+	+	+
m26	*E. gallinarum*	+	-	+	+	-	+
M43	*E. gallinarum*	+++	-	+	+	+	-
w13	*E. gallinarum*	+++	-	+	+	+	+
q27	*E. gallinarum*	++	-	+	-	-	+
c65	*E. gallinarum*	+	+	+	-	-	+
c101	*E. gallinarum*	++	+	+	-	+	-
CC8	*E. gallinarum*	++	-	-	+	+	-
CC23	*E. gallinarum*	+	+	+	-	-	+
CC29	*E. gallinarum*	+	+	+	-	+	+
m25	*E. gallinarum*	+	+	+	-	-	+
m52	*E. gallinarum*	++	-	+	-	-	+
M76	*E. gallinarum*	+	-	+	-	+	-
c77	*E. gallinarum*	++	+	+	-	-	+
c115	*E. gallinarum*	+	+	-	-	-	+
CC18	*E. gallinarum*	+	-	-	-	-	+
f27	*E. gallinarum*	++	+	+	-	-	-
f31	*E. gallinarum*	++	+	-	-	-	+
f34	*E. gallinarum*	++	-	-	-	-	+
f38	*E. gallinarum*	+	+	-	-	-	+
m77	*E. gallinarum*	++	+	+	-	-	+
M3	*E. gallinarum*	++	+	+	+	-	-

M24	*E. gallinarum*	+++	+	+	-	+	-
M39	*E. gallinarum*	++	-	+	+	-	-
q36	*E. gallinarum*	+	+	-	-	-	+
c83	*E. gallinarum*	++	+	+	-	-	-
c88	*E. gallinarum*	+++	+	-	-	-	+
c56	*E. gallinarum*	++	+	-	-	-	-
m6	*E. gallinarum*	+	-	-	-	-	-
m39	*E. gallinarum*	+++	-	-	-	-	-
SR17	*E. gallinarum*	+	+	-	-	-	-
c54	*E. gallinarum*	++	+	-	-	-	-
m61	*E. gallinarum*	+++	+	-	-	-	-

Biofilm strength (based on OD value): non-biofilm forming/weak <0.120 (+)

moderately 0.120-0.240 (++)

strong >0.240 (+++)

c- chicken meat; q- quail meat; m-mutton; p- pork; f- fish; cb- carabeef; M-milk; w- water; UD- uterine discharges; h- human stool samples; hd- human diarrhoeic samples; u- human urine samples; CC- faecal swabs of chicken; SR-rectal swabs of sheep and BR-rectal swabs of buffalo

Out of 236 biofilm forming Enterococcus isolates, 44 strong biofilm producers (18 *E. faecalis*, 13 *E. faecium*, 9 *E. gallinarum* and 4 *E. casseliflavus*), 85 medium level biofilm producers (62 *E. faecalis*, 4 *E. faecium*, 14 *E. gallinarum* and 5 *E. casseliflavus*) and 107 weak biofilm producers (31 *E. faecalis*, 50 *E. faecium*, 18 *E. gallinarum* and 8 *E. casseliflavus*) was identified by MTP assay.

Table-26: Correlation between phenotypic virulence factors (gelatinase, slime layer and hemolysin) and virulence genes (*gelE*, *asa*, *cylA*) in *Enterococcus* spp.

Species	**Gelatinase (%)**	***gelE* (%)**	**Slime layer (%)**	***asa* (%)**	**Hemolysin (%)**	***cylA* (%)**
***E. faecalis* (278)**	67 (24.10)	85 (30.57)	155 (55.75)	70 (25.17)	146 (52.51)	31 (11.15)
***E. faecium* (179)**	44 (24.58)	45 (25.13)	48 (26.81)	56 (31.28)	79 (44.13)	20 (11.17)
***E. gallinarum* (103)**	21 (20.38)	33 (32.03)	9 (8.73)	33 (32.03)	71 (68.93)	12 (11.65)
***E. casseliflavus* (48)**	9 (18.75)	18 (37.5)	31 (64.58)	21 (43.75)	16 (33.33)	5 (10.41)
Total (608)	**141 (23.19)**	**181 (29.76)**	**243 (39.96)**	**180 (29.60)**	**312 (51.31)**	**68 (11.18)**

4.6.2 Virulotyping:

In the present study, all 608 *Enterococcus* isolates were subjected to virulotyping i.e. identification of combination of various virulence factor associated genes (*gelE*, *ace*, *efaA*, *asa*, *cylA* and *hyl*) where 38, 30, 21 and 15 virulotypes were identified for *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus* (Tables- 27, 28, 29 and 30), respectively. A total of 323 *Enterococcus* isolates were not having any virulent marker.

4.6.2.1 Virulotyping of *E. faecalis* isolates

Among 128 virulent *E. faecalis* isolates, maximum number of virulence genes were detected in 13 isolates which were belonging to virulotype Vf1. Lowest numbers of virulence associated genes (one gene each) were detected in 30 isolates belonging toVf33, Vf34, Vf35, Vf36, Vf37 and Vf38. The discriminatory power of virulotyping for *E. faecalis* was found to be 0.9541. Numerical index of virulotying discrimination was calculated by Simpson's index of diversity as per 3.14.5.

Table-27: Virulotypes of *E. faecalis* isolates

Sample ID	No. of isolates	Virulotype	Virulence genes	No. of genes
c9, c39, c110, f46, m16, p25, w1, w3, w17, w24, UD22, u4 and h24	13	Vf1	*gelE*, *ace*, *efaA*, *asa*, *cylA* and *hyl*	6
q1, c86, c136, m12, m13 and w25	6	Vf2	*gelE*, *ace*, *efaA*, *asa* and *hyl*	5
f26	1	Vf3	*gelE*, *ace*, *efaA*, *cylA* and *hyl*	5
q14, c1, c17 and c58	4	Vf4	*gelE*, *ace*, *efaA*, *asa* and *hyl*	5
q 51, c151, CC21 and w9	4	Vf5	*gelE*, *ace*, *efaA* and *hyl*	4
c21, m28 and hd2	3	Vf6	*gelE*, *ace*, *asa* and *hyl*	4
f2	1	Vf7	*ace*, *efaA*, *cylA* and *hyl*	4
f23	1	Vf8	*gelE*, *asa*, *cylA* and *hyl*	
m17	1	Vf9	*gelE*, *efaA*, *cylA* and *hyl*	
P11 and u10	2	Vf10	*gelE*, *ace*, *efaA* and *cylA*	4
h4	1	Vf11	*gelE*, *ace*, *efaA* and *asa*	4
h5	1	Vf12	*gelE*, *efaA*, *asa* and *hyl*	4
c98	1	Vf13	*Ace*, *efaA*, *asa* and *cylA*	4
c24, c114 and hd3	3	Vf14	*gelE*, *asa* and *hyl*	3
c28, h10 and hd6	3	Vf15	*gelE*, *ace* and *efaA*	3

c106	1	Vf16	*gelE, cylA* and *hyl*	3
c108	1	Vf17	*gelE, ace* and *cylA*	3
f15	1	Vf18	*asa, cylA* and *hyl*	3
m19	1	Vf19	*gelE, ace* and *hyl*	3
m27 and M1	2	Vf20	*gelE, efaA* and *hyl*	3
m73	1	Vf21	*ace, asa* and *cylA*	3
p27 and UD13	2	Vf22	*gelE, asa* and *cylA*	3
q10, q32, q37, q43, q45, c73, c91, f53, m75, fb6, fc12, w11, UD18, u9, h30 and h36	16	Vf23	*gelE* and *asa*	2
q35, f22 and f51	3	Vf24	*asa* and *hyl*	2
q41	1	Vf25	*ace* and *efaA*	2
c107, SR12, CC19, SR26, CC15, p6 and h40	7	Vf26	*gelE* and *ace*	2
c118	1	Vf27	*gelE* and *cylA*	2
c137, BR5, SR24, w2, h11 and h29	6	Vf28	*gelE* and *efaA*	2
f11, m2, m9, m34, m41 and m47	6	Vf29	*ace* and *Asa*	2
f33 and M50	2	Vf30	*gelE* and *hyl*	2
M71	1	Vf31	*cyla* and *efaA*	2
UD12	1	Vf32	*cylA* and *hyl*	2
c6, c14, c19, CC9, SR22, h33 and h41	7	Vf33	*gelE*	1
c7, c10, c20, m29 and h1	5	Vf34	*ace*	1
m22	1	Vf35	*efaA*	1
c72, c87, f6, CC17, cb39, w20, UD16 and h39	8	Vf36	*asa*	1
c8 and c104	2	Vf37	*cylA*	1
cb7, p44, w15, w19, u1, u5 and u6	7	Vf38	*hyl*	1
Number of unrelated strains : 128 Number of types : 38 Discriminatory power : 0.9541				

4.6.2.2 Virulotyping of *E. faecium* isolates

Among 83 virulent *E. faecium* isolates, maximum number of virulence genes were detected in 4 isolates belonging to virulotype VTf1. Lowest numbers of virulence associated genes (one gene each) were detected in 25 isolates belongs toVTf26, VTf27, VTf28, VTf29 and VTf30. The discriminatory power of virulotyping for *E. faecium* was

found to be 0.9521. Numerical index of virulotying discrimination was calculated by Simpson's index of diversity as per 3.14.5.

Table-28: Virulotypes of *E. faecium* isolates

Sample ID	No of isolates	Virulotype	Virulence genes	No of genes
c76, c84, CC6 and UD23	4	VTf1	*gelE*, *ace*, *efaA*, *asa*, *cylA* and *hyl*	6
c47,f41 and h25	3	VTf2	*gelE*, *ace*, *efaA*, *asa* and *hyl*	5
m10 and m42	2	VTf3	*gelE*, *ace*, *asa*, *cylA* and *hyl*	5
M45 and p14	2	VTf4	*gelE*, *efaA*, *asa*, *hyl* and *cylA*	5
w6	1	VTf5	*gelE*, *efaA*, *asa*, *cylA* and *hyl*	5
q11, c62 and m51	3	VTf6	*ace*, *efaA*, *asa* and *hyl*	4
c81	1	VTf7	*ace*, *efaA*, *cylA* and *hyl*	4
c111	1	VTf8	*gelE*, *ace*, *efaA* and *hyl*	4
c154	1	VTf9	*gelE*, *asa*, *cylA* and *hyl*	4
f14 and p32	2	VTf10	*gelE*, *ace*, *efa* and *asa*	4
w8 and UD19	2	VTf11	*gelE*, *ace*, *asa* and *hyl*	4
q13	1	VTf12	*ace*, *efaA* and *asa*	3
q24, c64, M36, p5 and w12	5	VTf13	*gelE*, *asa* and *hyl*	3
c59	1	VTf14	*ace*, *efaA* and *asa*	3
m3, m35 and m48	3	VTf15	*gelE*, *cylA* and *hyl*	3
p16	1	VTf16	*gelE*, *asa* and *cylA*	3
p54 and w21	2	VTf17	*gelE*, *ace* and *efaA*	3
c15	1	VTf18	*gelE* and *Ace*	2
c57, c75, c80, m82, BR7, cb1, cb4, cb24 and h31	9	VTf19	*gelE* and *asa*	2
c71, c156, f18, f30, f40, m1, m31 and UD9	8	VTf20	*asa* and *hyl*	2
c74	1	VTf21	*ace* and *efaA*	2
c135	1	VTf22	*cylA* and *hyl*	2
c144	1	VTf23	*gelE* and *cylA*	2
f39	1	VTf24	*asa* and *cylA*	2
w22	1	VTf25	*gelE* and *efaA*	2
q6, q8, c55, f1, f17, SR23, p49, UD4, h26 and h42	10	VTf26	*Asa*	1
c103 and c123	2	VTf27	*cylA*	1
m11, m44, SR4, cb35 and cb54	5	VTf28	*gelE*	1
m53, M63 and p21	3	VTf29	*ace*	1
SR13, p34, p39, h7 and h8	5	VTf30	*hyl*	1
Number of unrelated strains : 83 Number of types : 30 Discriminatory power : 0.9521				

4.6.2.3 Virulotyping of *E. gallinarum* isolates

Among 49 virulent *E. gallinarum* isolates, maximum number of virulence genes were detected in 6 isolates belonging to virulotype VTg1. Lowest numbers of virulence associated genes (one gene each) were detected in 14 isolates belongs toVTg18, VTg19, VTg20 and VTg21. The discriminatory power of virulotyping for *E. gallinarum* was found to be 0.9371. Numerical index of virulotying discrimination was calculated by Simpson's index of diversity as per 3.14.5.

Table-29: Virulotypes of *E. gallinarum* isolates

Sample ID	No of isolates	Virulotype	Virulence genes	No of genes
c3, c33, c99, p36, UD10 and h20	6	VTg1	*gelE*, *ace*, *efa*, *asa*, *cylA* and *hyl*	6
c31 and h17	2	VTg2	*gelE*, *ace*,*efaA*, *asa* and *hyl*	5
c79	1	VTg3	*gelE*, *ace*, *efaA* and *hyl*	4
c112 and M43	2	VTg4	*gelE*, *ace*, *efaA* and *cylA*	4
c120	1	VTg5	*gelE*, *asa*, *cylA* and *hyl*	4
c159 and w13	2	VTg6	*gelE*, *ace*, *efaA* and *asa*	4
m26	1	VTg7	*gelE*, *ace*, *asa* and *hyl*	4
q27, c65, CC13, m25 and m52	5	VTg8	*gelE*, *asa* and *hyl*	3
c101 and M76	2	VTg9	*gelE*, *efaA* and *cylA*	3
CC8	1	VTg10	*Ace*, *efaA* and *Hyl*	3
c192	1	VTg11	*gelE* , *efaA* and *asa*	3
c77 and m77	2	VTg12	*gelE* and *asa*	2
c115, CC10, f31, f34 and f38	5	VTg13	*Asa* and *hyl*	2
f27	1	VTg14	*gelE* and *Hyl*	2
M3 and M39	2	VTg15	*gelE* and *ace*	2
M24	1	VTg16	*gelE* and *efaA*	2
q36, c88, CC31, m5, m36, SR29, b21 and M4	8	VTg17	*Asa*	1
c83, c153, f29 and m67	4	VTg18	*gelE*	1
c127	1	VTg19	*cylA*	1
w18	1	VTg20	*Hyl*	1
Number of unrelated strains : 49 Number of types : 20 Discriminatory power : 0.9362				

4.6.2.4 Virulotyping of *E. casseliflavus* isolates

Among 25 virulent *E. casseliflavus* isolates, maximum number of virulence genes was detected in 2 isolates belonging to virulotype VTc1. Lowest numbers of virulence associated genes (one gene each) were detected in 6 isolates belonging toVTc14 and VTg15. The discriminatory power of virulotyping for *E. casseliflavus* was found to be 0.94. Numerical index of virulotying discrimination was calculated by Simpson's index of diversity as per 3.14.5.

Table-30: Virulotypes of *E. casseliflavus* isolates

Sample ID	No of isolates	Virulotype	Virulence genes	No of genes
q44 and c141	2	VTc1	*gelE*, *ace*, *efaA*, *asa*, *cylA* and *hyl*	6
c119	1	VTc2	*gelE*, *ace*, *efaA*, *asa* and *cylA*	5
c13, m72 and w10	3	VTc3	*gelE*, *ace*, *efaA*, *asa* and *hyl*	5
c142	1	VTc4	*gelE*, *efaA*, *asa*, *cylA* and *hyl*	5
c93	1	VTc5	*ace*, *efaA*, *asa* and *hyl*	4
m30	1	VTc6	*gelE*, *efaA*, *asa* and *hyl*	4
c122	1	VTc7	*gelE*, ace and efaA	4
SR1 and CC16	2	VTc8	*gelE*, *asa* and *hyl*	3
M47	1	VTc9	*ace*, *asa* and *cylA*	3
c32, c66 and f35	3	VTc10	*gelE* and *asa*	2
p17	1	VTc11	*gelE* and *hyl*	2
p55	1	VTc12	*gelE* and *efaA*	2
h19	1	VTc13	*gelE* and *asa*	2
c56, m6, m39, SR17 and M8	5	VTc14	*Asa*	1
SR3	1	VTc15	*gelE*	1
Number of unrelated strains : 25 Number of types : 15 Discriminatory power : 0.94				

4.7 ANTIBIOGRAM STUDIES

All the 608 *Enterococcus* spp. isolates were subjected to antibiotic sensitivity test using 16 different most commonly used antibiotics in Veterinary medicine (Table-10). The antimicrobial agents from different classes were taken in order to know the multidrug resistance in *Enterococcus* isolates. All *Enterococcus* isolates showed high rate of susceptibility to ampilcillin (90.78%) followed by piperacillin (81.90%), linezolid (78.94%), teicoplanin (78.61%), penicillin-G (76.48%), rifampicin (75.16%), vancomycin (74.67%), nitrofurantoin (74.67%), chloramphenicol (71.71%), gentamicin (66.77%), tetracycline (48.19%), ciprofloxacin (47.86%), streptomycin (44.24%), erythromycin (19.73%), ceftazidime (6.74%) and polymixin (4.44%). Notable percentage of isolates were intermediately resistant against ciprofloxacin (21.05%) followed by tetracycline (17.10%), streptomycin (11.67%), chloramphenicol (11.51%), teicoplanin (10.85%), nitrofurantoin (10.36%), linezolid (10.03%), piperacillin (9.21%), gentamicin (6.74%), vancomycin (6.08%), rifampicin (3.61%), erythromycin (2.63%) and penicillin-G (0.16%). None of the isolates showed intermediate resistance to ampicillin, ceftazidime and polymixin-B. Among 608 *Enterococcus* isolates, higher resistance was observed towards polymixin-B (95.55%) followed by ceftazidime (93.25%), erythromycin (77.63%), streptomycin (44.07%), tetracycline (34.70%), ciprofloxacin (31.08%), gentamicin (26.48%), penicillin-G (23.35%), rifampicin (21.21%), vancomycin (19.24%), chloramphenicol (16.77%), nitrofurantoin (14.80%), linezolid (11.01%), teicoplanin (10.52%), ampicillin (9.21%) and piperacillin (8.88%). Species and source wise, antibiotic resistance patterns were given in detail in Tables- 31 to 34 and Fig- 25 to 31.

Out of 278 *E. faecalis* isolates, highest resistance was found against polymixin-B (94.60%) followed by ceftazidime (93.16%), erythromycin (84.17%), streptomycin (50.35%), tetracycline (41.36%), gentamicin (37.41%), ciprofloxacin (31.65%),

rifampicin (30.21%), penicillin-G (23.38%), vancomycin (21.22%), chloramphenicol (20.86%), nitrofurantoin (18.70%), teicoplanin (12.58%) linezolid (10.43%), ampilcillin (10.07%) and piperacillin (8.99%).

The 179 *E. faecium* isolates have shown highest resistance towards polymixin-B (95.53%) followed by ceftazidime (87.70%), erythromycin (79.88%), streptomycin (35.19%), tetracycline (29.05%), ciprofloxacin (30.16%), penicillin-G (21.22%), gentamicin (20.11%), rifampicin (15.64%), vancomycin (14.52%), nitrofurantoin (13.96%), chloramphenicol and linezolid (11.73% each), piperacillin (10.61%), ampilcillin (8.37%) and teicoplanin (5.02%).

Out of 103 *E. gallinarum* isolates, highest resistance was found against ceftazidime (100%) followed by polymixin-B (96.11%), erythromycin (63.10%), streptomycin (44.66%), tetracycline (37.86%), penicillin-G (32.03%), ciprofloxacin (30.09%), gentamicin (18.44%), chloramphenicol (16.5%), vancomycin (15.53%), linezolid (14.56%), rifampicin and teicoplanin (11.65% each), ampilcillin (10.67%), nitrofurantoin (6.79%) and piperacillin (2.91%).

Out of 48 *E. casseliflavus* isolates, highest resistance was found against polymixin-B and ceftazidime (100% each) followed by erythromycin (62.5%), streptomycin (39.58%), ciprofloxacin and vancomycin (33.33% each), teicoplanin (8/48), piperacillin (14.58%) chloramphenicol, nitrofurantoin and penicillin-G (12.50% each), rifampicin and tetracycline (10.41% each), ampicillin, gentamicin and linezolid (4.16% each).

Table 31: Antibiotic resistance/sensitivity pattern among *Enterococcus* isolates by disc diffusion method

S.No	Antibiotic disc	Pattern of antibiogram					
		Sensitive		Intermediate		Resistant	
		No	%	No	%	No	%
1	Ampicillin (AMP- 10µg)	552	90.78%	0	0	56	9.21%
2	Ceftazidime (CAZ- 30µg)	41	6.74%	0	0	567	93.25%
3	Chloramphenicol (C- 30µg)	436	71.71%	70	11.51%	102	16.77%
4	Ciprofloxacin (CIP- 5µg)	291	47.86%	128	21.05%	189	31.08%
5	Erythromycin (E- 15µg)	120	19.73%	16	2.63%	472	77.63%
6	Gentamicin (GEN-10µg)	406	66.77%	41	6.74%	161	26.48%
7	Linezolid (LZ- 30µg)	480	78.94%	61	10.03%	67	11.01%
8	Nitrofurantoin (NIT- 300µg)	454	74.67%	64	10.36%	90	14.80%
9	Penicillin-G (P- 10U)	465	76.48%	1	0.16%	142	23.35%
10	Piperacillin (PI- 100µg)	498	81.90%	56	9.21%	54	8.88%
11	Polymixin B (PB- 300U)	27	4.44%	0	0	581	95.55%
12	Rifampicin (RIF- 5µg)	457	75.16%	22	3.61%	129	21.21%
13	Streptomycin (S- 300µg)	269	44.24%	71	11.67%	268	44.07%
14	Teicoplanin (TEI- 30µg)	478	78.61%	66	10.85%	64	10.52%
15	Tetracycline (TE- 30µg)	293	48.12%	104	17.10%	211	34.70%
16	Vancomycin (VA- 30µg)	454	74.67%	37	6.08%	117	19.24%

Table-32: Antibiotic resistance among different *Enterococcus* species by disc diffusion method

Antibiotic disc	*E. faecalis* (n= 278)	*E. faecium* (n= 179)	*E. gallinarum* (n= 103)	*E. casseliflavus* (n= 48)
Ampicillin (AMP- 10µg)	28 (10.07%)	15 (8.37%)	11 (10.67%)	2 (4.16%)
Ceftazidime (CAZ- 30µg)	259 (93.16%)	157 (87.70%)	103 (100%)	48 (100%)
Chloramphenicol (C- 30µg)	58 (20.86%)	21 (11.73%)	17 (16.50%)	6 (12.50%)
Ciprofloxacin (CIP- 5µg)	88 (31.65%)	54 (30.16%)	31 (30.09%)	16 (33.33%)
Erythromycin (E- 15µg)	234 (84.17%)	143 (79.88%)	65 (63.10%)	30 (62.5%)
Gentamicin (GEN- 10µg)	104 (37.41%)	36 (20.11%)	19 (18.44%)	2 (4.16%)
Linezolid (LZ- 30µg)	29 (10.43%)	21(11.73%)	15 (14.56%)	2 (4.16%)
Nitrofurantoin (NIT- 300µg)	52 (18.70%)	25 (13.96%)	7 (6.79%)	6 (12.5%)
Penicillin-G (P- 10U)	65 (23.38%)	38 (21.22%)	33 (32.03%)	6 (12.50%)
Piperacillin (PI- 100µg)	25 (8.99%)	19 (10.61%)	3 (2.91%)	7 (14.58%)
Polymixin B (PB- 300U)	263 (94.60%)	171 (95.53%)	99 (96.11%)	48 (100%)
Rifampicin (RIF- 5µg)	84 (30.21%)	28 (15.64%)	12 (11.65%)	5 (10.41%)
Streptomycin (S- 30µg)	140 (50.35%)	63 (35.19%)	46 (44.66%)	19 (39.58%)
Teicoplanin (TEI- 30µg)	35 (12.58%)	9 (5.02%)	12 (11.65%)	8 (16.66%)
Tetracycline (TE- 30µg)	115 (41.36%)	52 (29.05%)	39 (37.86%)	5 (10.41%)
Vancomycin (VA- 30µg)	59 (21.22%)	26 (14.52%)	16 (15.53%)	16 (33.33%)

Table-33: Antibiotic susceptibility/resistance among different *Enterococcus* species

Antibiotic disc	*E. faecalis* (n= 278)			*E. faecium* (n= 179)			*E. gallinarum* (n= 103)			*E. casseliflavus* (n= 48)		
	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
AMP	250 (89.92%)	0	28 (10.07%)	164 (91.62%)	0	15 (8.37%)	92 (89.32%)	0	11 (10.67%)	46 (95.83%)	0	2 (4.16%)
CAZ	19 (6.83%)	0	259 (93.16%)	22 (12.29%)	0	157 (87.70%)	0	0	103 (100%)	0	0	48 (100%)
C	196 (70.50%)	24 (8.63%)	58 (20.86%)	131 (73.18%)	27 (15.08%)	21 (11.73%)	74 (71.84%)	12 (11.65%)	17 (16.50%)	35 (72.91%)	7 (14.58%)	6 (12.50%)
CIP	146 (52.51%)	44 (15.82%)	88 (31.65%)	75 (41.89%)	50 (27.93%)	54 (30.16%)	54 (52.42%)	18 (17.47%)	31 (30.09%)	16 (33.33%)	16 (33.33%)	16 (33.33%)
E	43 (15.46%)	1 (0.35%)	234 (84.17%)	35 (19.55%)	1 (0.55%)	143 (79.88%)	25 (24.27%)	13 (12.62%)	65 (63.10%)	17 (35.41%)	1 (2.08%)	30 (62.50%)
GEN	153 (55.03%)	21 (7.55%)	104 (37.41%)	133 (74.30%)	10 (5.58%)	36 (20.11%)	78 (75.72%)	6 (5.82%)	19 (18.44%)	42 (87.50%)	4 (8.33%)	2 (4.16%)
LZ	215 (77.33%)	34 (12.23%)	29 (10.43%)	155 (86.59%)	3 (1.67%)	21 (11.73%)	71 (68.93%)	17 (16.50%)	15 (14.56%)	39 (81.25%)	7 (14.58%)	2 (4.16%)
NIT	194 (69.78%)	32 (11.51%)	52 (18.70%)	144 (80.44%)	9 (5.02%)	25 (13.96%)	82 (79.61%)	14 (13.59%)	7 (6.79%)	34 (70.83%)	8 (16.66%)	6 (12.50%)
P	213 (76.61%)	0	65 (23.38%)	141 (78.77%)	0	38 (21.22%)	70 (67.96%)	0	33 (32.03%)	41 (85.41%)	1 (2.08%)	6 (12.50%)
PI	221 (79.49%)	32 (11.51%)	25 (8.99%)	149 (83.24%)	11 (6.14%)	19 (10.61%)	87 (84.46%)	13 (12.62%)	3 (2.91%)	41 (85.41%)	0	7 (14.58%)
PB	15 (5.39%)	0	263 (94.60)	8 (4.46%)	0	171 (95.53)	4 (3.88%)	0	99 (96.11%)	0	0	48 (100%)
RIF	185 (66.54%)	9 (3.23%)	84 (30.21%)	148 (82.68%)	3 (1.67%)	28 (15.64%)	84 (81.55%)	7 (6.79%)	12 (11.65%)	40 (83.33%)	3 (6.25%)	5 (10.41%)
S	101 (36.33%)	37 (13.30%)	140 (50.35%)	99 (55.30%)	17 (9.49%)	63 (35.19%)	48 (46.60%)	9 (8.73%)	46 (44.66%)	21 (43.75%)	8 (16.66%)	19 (39.58%)
TEI	213 (76.61%)	30 (10.79%)	35 (12.58%)	151 (84.35%)	19 (10.61%)	9 (5.02%)	76 (73.78%)	15 (14.56%)	12 (11.65%)	38 (79.16%)	2 (4.16%)	8 (16.66%)
TE	110 (39.56%)	53 (19.06%)	115 (41.36%)	105 (58.65%)	22 (12.29%)	52 (29.05%)	44 (42.71%)	20 (19.41%)	39 (37.86%)	34 (70.83%)	9 (18.75%)	5 (10.41%)
VA	206 (74.10%)	13 (4.67%)	59 (21.22%)	141 (78.77%)	12 (6.70%)	26 (14.52%)	82 (79.61%)	5 (4.85%)	16 (15.53%)	25 (52.08%)	7 (14.58%)	16 (33.33%)

Table-34: Antibiotic resistance pattern of *Enterococcus* spp. isolated from different sources

Samples (No. of Enterococcus isolates)	AMP (%)	CAZ (%)	C (%)	CIP (%)	E (%)	GEN(%)	LZ (%)	NIT (%)	P (%)	PI (%)	PB (%)	RIF (%)	S (%)	TEI (%)	TE (%)	VA (%)
FOODS OF ANIMAL ORIGIN																
c (142)	23 (16.19)	130 (91.54)	40 (28.16)	81 (57.04)	112 (78.87)	27 (19.01)	14 (9.85)	36 (25.35)	46 (32.39)	14 (9.85)	132 (92.95)	32 (22.53)	67 (47.18)	9 (6.33)	75 (52.81)	19 (13.38)
q (52)	0	52 (100)	8 (15.38)	11 (21.15)	38 (73.07)	26 (50.00)	0	0	27 (51.92)	3 (5.76)	52 (100)	9 (17.30)	23 (44.23)	8 (15.38)	19 (36.53)	12 (23.07)
m (44)	0	36 (81.81)	4 (9.09)	7 (15.90)	40 (90.90)	26 (59.09)	0	20 (45.45)	9 (20.45)	0	44 (100)	11 (25.00)	20 (45.45)	10 (22.72)	15 (34.09)	25 (56.81)
p (46)	11 (23.91)	46 (100)	13 (28.26)	13 (28.26)	38 (82.60)	4 (8.69)	0	6 (13.04)	7 (15.21)	6 (13.04)	46 (100)	22 (47.82)	27 (58.69)	2 (4.34)	25 (54.34)	9 (19.56)
f (47)	3 (6.38)	47 (100)	14 (29.78)	10 (21.27)	43 (91.48)	26 (55.31)	0	0	14 (29.78)	1 (2.12)	47 (100)	19 (40.42)	28 (59.57)	13 (27.65)	20 (42.55)	6 (12.76)
cb (45)	0	45 (100)	0	6 (13.33)	30 (66.66)	3 (6.66)	0	0	0	0	45 (100)	0	4 (8.88)	0	2 (4.44)	7 (15.55)
M (69)	4 (5.79)	60 (86.95)	2 (2.89)	16 (23.18)	61 (88.40)	22 (31.88)	9 (13.04)	5 (7.24)	2 (2.89)	14 (20.28)	69 (100)	8 (11.59)	35 (50.72)	11 (15.94)	7 (10.14)	6 (8.69)
ANIMAL FAECAL SWABS																
CC (23)	1 (4.34)	21 (91.30)	2 (8.69)	2 (9.52)	16 (76.19)	2 (9.52)	2 (9.52)	0	6 (28.57)	1 (4.16)	21 (100)	1 (4.76)	7 (33.33)	1 (4.76)	2 (9.52)	5 (23.80)
SR (23)	4 (17.39)	23 (100)	7 (30.43)	6 (26.08)	15 (65.21)	8 (34.78)	2 (8.69)	5 (21.73)	5 (21.73)	0	21 (91.30)	0	14 (60.86)	0	3 (13.04)	6 (26.08)
BR (16)	5 (31.25)	10 (62.5)	1 (6.25)	12 (75.00)	7 (43.75)	3 (18.75)	0	4 (25.00)	0	0	12 (75.00)	0	2 (12.50)	0	2 (12.50)	4 (25.00)
PR (11)	1 (9.09)	7 (63.63)	0	2 (18.18)	3 (27.27)	4 (36.36)	2 (18.18)	3 (27.27)	3 (27.27)	0	7 (63.63)	0	11 (100)	4 (36.36)	8 (72.72)	2 (18.18)
HUMAN SAMPLES																
h (36)	2 (5.55)	36 (100)	0	5 (13.88)	24 (66.66)	4 (11.11)	19 (52.77)	0	9 (25.00)	0	36 (100)	0	3 (8.33)	0	8 (22.22)	8 (22.22)
hd (12)	2 (16.66)	12 (100)	5 (41.66)	4 (33.33)	11 (78.57)	1 (8.33)	11 (91.66)	2 (16.66)	3 (25.00)	2 (16.66)	12 (100)	5 (41.66)	4 (33.33)	0	5 (41.66)	1 (8.33)
u (6)	0	6 (100)	1 (16.66)	3 (50.00)	6 (100)	0	2 (33.33)	6 (100)	4 (66.66)	6 (100)	5 (83.33)	4 (66.66)	3 (50.00)	0	3 (50.00)	2 (33.33)
ENVIRONMENTAL SAMPLES																
w (22)	0	22 (100)	5 (22.72)	5 (22.72)	17 (77.27)	3 (13.63)	4 (19.18)	0	3 (13.63)	3 (13.63)	22 (100)	10 (45.45)	12 (54.54)	6 (27.27)	9 (40.90)	2 (9.09)
MISCELLANEOUS																
UD (14)	0	14 (100)	0	6 (42.85)	11 (78.57)	2 (14.28)	2 (19.18)	3 (21.42)	4 (28.57)	4 (28.57)	10 (71.42)	8 (57.14)	8 (57.14)	0	8 (57.14)	3 (21.42)
Total (608)	**56 (9.21)**	**567 (93.25)**	**102 (16.77)**	**189 (31.08)**	**472 (77.63)**	**161 (26.48)**	**67 (11.01)**	**90 (14.80)**	**142 (23.35)**	**54 (8.88)**	**581 (95.55)**	**129 (21.21)**	**268 (44.07)**	**64 (10.52)**	**211 (34.70)**	**117 (19.24)**

c-chicken meat; q-quail meat; m-mutton; p- pork; f- fish; cb- carabeef; M- milk; w- water; UD- uterine discharges; h- human stool samples; hd- human diarrhoeic samples; u- human urine samples; CC- cloacal swabs of chicken; SR- rectal swabs of sheep; BR- rectal swabs of buffalo and PR- rectal swabs of pig

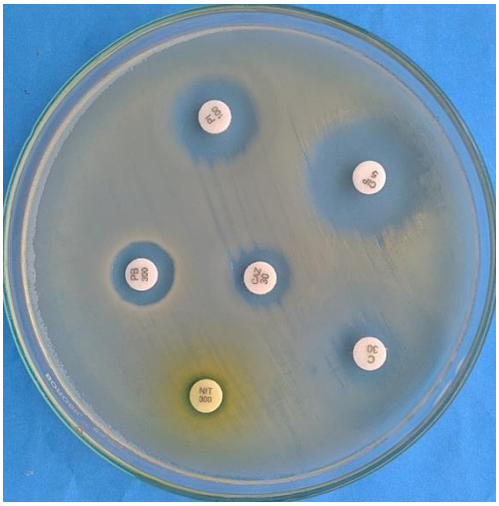


Fig-25: Plate showing antibiogram of *E. faecalis*

Piperacillin (100µg)- Resistant

Ciprofloxacin (5µg)- Sensitive

Polymixin B (300U)- Resistant

Ceftazidime (30µg)- Resistant

Chloramphenicol (30µg)- Resistant

Nitrofurantoin (300µg)- Resistant

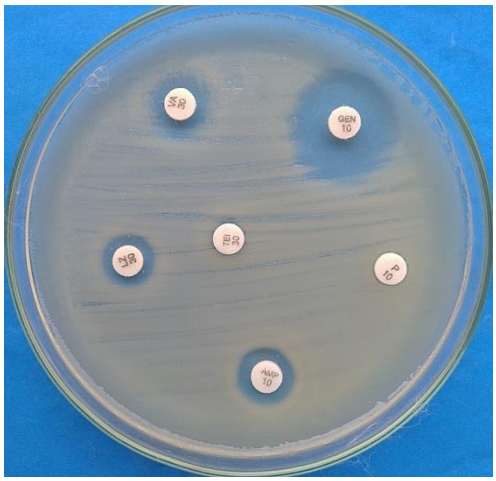


Fig-26: Plate showing antibiogram of *E. faecalis*

Vancomycin (30µg)- Resistant

Gentamicin (10µg)- Sensitive

Teicoplanin (30µg)- Resistant

Linezolid (30µg)- Resistant

Penicillin-G (10units)- Resistant

Ampicillin (10µg)- Resistant

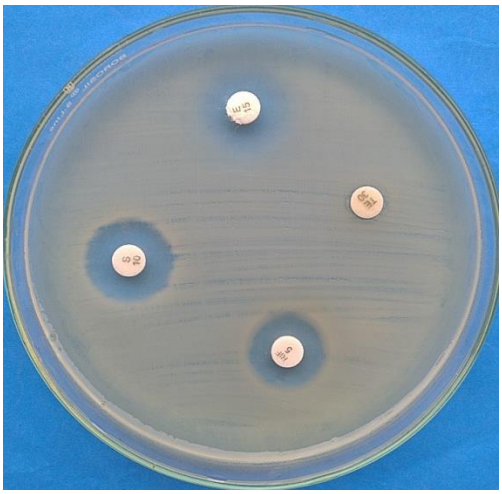


Fig-27: Plate showing antibiogram of *E. faecalis*

Erythromycin (15µg)- Resistant

Tetracycline (30µg)- Resistant

Rifampicin (5µg)- Resistant

Streptomycin (10µg)- Sensitive

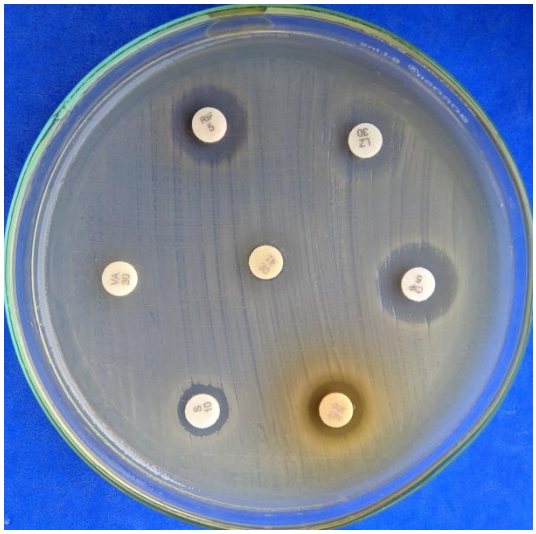


Fig-28: Plate showing antibiogram of *E. faecium*

Rifampicin (5µg)- Intermediate

Ciprofloxacin (5µg)- Sensitive

Nitrofurantoin (300µg)- Resistant

Streptomycin (10µg)- Resistant

Vancomycin (30µg)- Resistant

Tetracycline (30µg)- Resistant

Linezolid (30µg)- Intermediate

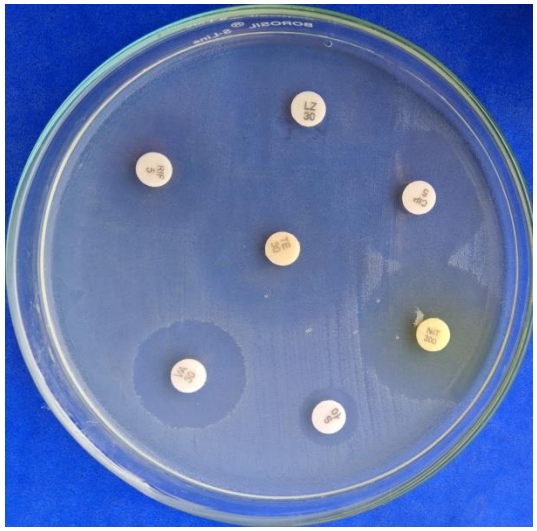


Fig-29: Plate showing antibiogram of *E. gallinarum*

Ciprofloxacin (5µg)- Sensitive

Rifampicin (5µg)- Sensitive

Tetracycline (30µg)- Sensitive

Streptomycin (10µg)- Resistant

Vancomycin (30µg)- Sensitive

Linezolid (30µg)- Sensitive

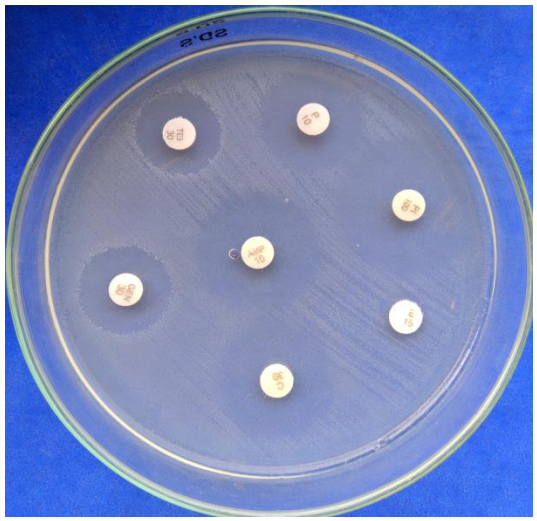


Fig-30: Plate showing antibiogram of *E. casseliflavus*

Gentamicin (10µg)- Sensitive

Teicoplanin (30µg)- Sensitive

Penicillin-G (10units)- Sensitive

Ampicillin (10µg)- Sensitive

Erythromycin (15µg)- Resistant

Piperacillin (100µg)- Sensitive

Chloramphenicol (30µg)- Resistant

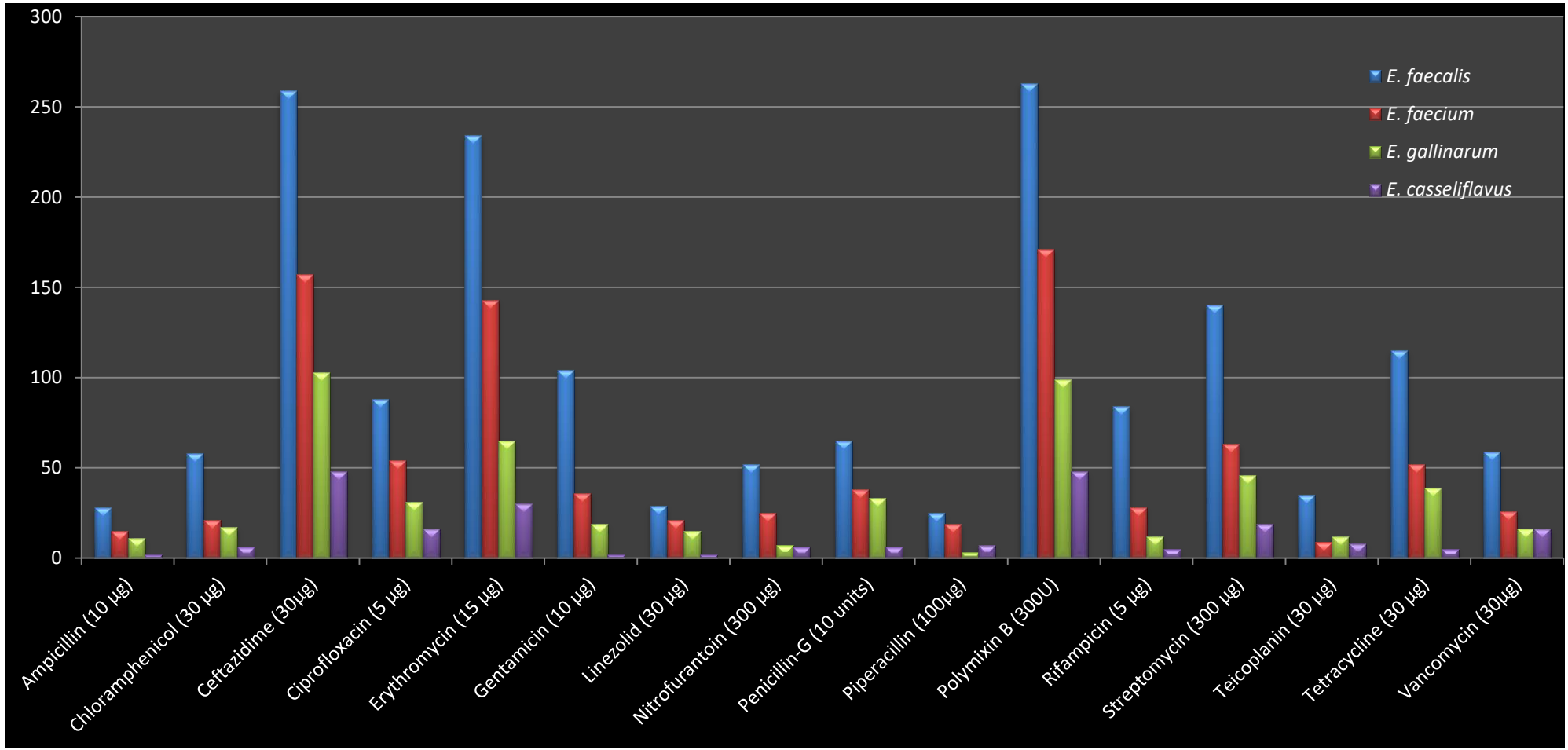


Fig-31: Bar diagram showing antibiotic resistance patterns of different *Enterococcus* species

4.7.1 Molecular detection of HLAR genes in *Enterococcus* spp. By PCR

The *Enterococcus* isolates which exhibited phenotypic resistance to either gentamicin/streptomycin were subjected to HLAR phenotypic detection. All the isolates which exhibited resistance for either of the aminoglycosides i.e. gentamicin/ kanamycin/ streptomycin/ amikacin were selected for molecular detection of aminoglycoside resistance genes. (Fig-32) was selected for molecular detection of HLAR genes. Out of 271 isolates that were phenotypically resistant to aminoglycosides, 179 (66.05%) isolates were positive for HLAR genes and *aac(6´)Ie-aph(2˝)Ia* was the only gene detected in all isolates. Among the 608 *Enterococcus* isolates, 95 (15.62%) *E. faecalis* isolates, 44 *E. faecium* (7.23%), 30 *E. gallinarum* (4.93%) isolates and 10 (1.64%) *E. casseliflavus* were carrying *aac(6´)Ie-aph(2˝)Ia* gene (Table- 35 and 38 and Fig-33).

4.7.2 Molecular detection of β-lactamase gene (*blaZ*) by PCR

Out of 608 *Enterococcus* isolates, 175 (33 ampicillin resistant only, 119 penicillin resistant only and 23 both ampicillin and penicillin resistant isolates) were found to be resistant to either penicillin or ampicillin or both and were subjected to detection of *blaZ* gene by PCR. Out of 175 isolates, 127 (72.57%) isolates were positive for *blaZ* gene (Table-36 and 38 and Fig-34). The *blaZ* gene was predominantly detected in *E. faecium* (62/127, 48.81%), followed by *E. faecalis* (40/127, 31.49%), *E. gallinarum* (17/127, 13.38%) and *E. casseliflavus* (8/127, 6.29%).

TABLE-35: Distribution of HLAR genes in different *Enterococcus* spp. isolated from different sources

Samples (No. of HLAR positive isolates)	*E. faecalis*	*E. faecium*	*E. gallinarum*	*E. casseliflavus*
FOODS OF ANIMAL ORIGIN				
Chicken (45)	26 (57.77%)	4 (8.88%)	9 (20.00%)	6 (13.33%)
Quail (23)	8 (34.78%)	13 (56.52%)	2 (8.69%)	0
Mutton (18)	14 (77.77%)	0	0	4 (22.22%)
Pork (18)	7 (38.88%)	11 (61.11%)	0	0
Fish (15)	9 (60.00%)	0	6 (40.00%)	0
Carabeef (8)	0	8 (100%)	0	0
Milk (0)	0	0	0	0
TOTAL (127)	**64 (50.39%)**	**36 (28.34%)**	**17 (13.38%)**	**10 (7.87%)**
ANIMAL FAECAL SWABS				
Chicken cloacal swabs (12)	7 (58.33%)	0	5 (41.66%)	0
Sheep rectal swabs (6)	5 (83.33%)	0	1 (16.66%)	0
Buffalo rectal swabs (5)	2 (40.00%)	0	3 (60.00%)	0
Pig rectal swabs (4)	3 (75.00%)	0	1 (25.00%)	0
TOTAL (27)	**17 (62.96%)**	**0**	**10 (37.03%)**	**0**
HUMAN SAMPLES				
Human Stool samples (17)	7 (41.11%)	7 (41.17%)	3 (17.64%)	0
Human diarrhoeic stool samples (0)	0	0	0	0
Human urine samples (2)	2 (100%)	0	0	0
TOTAL (19)	**9 (47.36%)**	**7 (36.84%)**	**3 (15.78%)**	**0**
ENVIRONMENTAL SAMPLES				
Water (3)	2 (66.66%)	1 (33.33%)	0	0
MISCELLANEOUS				
Uterine discharges (3)	3 (100%)	0	0	0
Total (179)	**95 (53.07%)**	**44 (24.58%)**	**30 (16.75%)**	**10 (5.58%)**

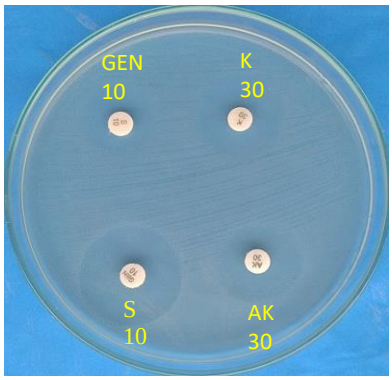


Fig-32: Plate showing phenotypic detection of HLAR in *Enterococcus* spp.

Kanamycin (30µg)- Resistant

Gentamicin (10µg)- Sensitive

Streptomycin (10µg)- Resistant

Amikacin (30µg)- Sensitive

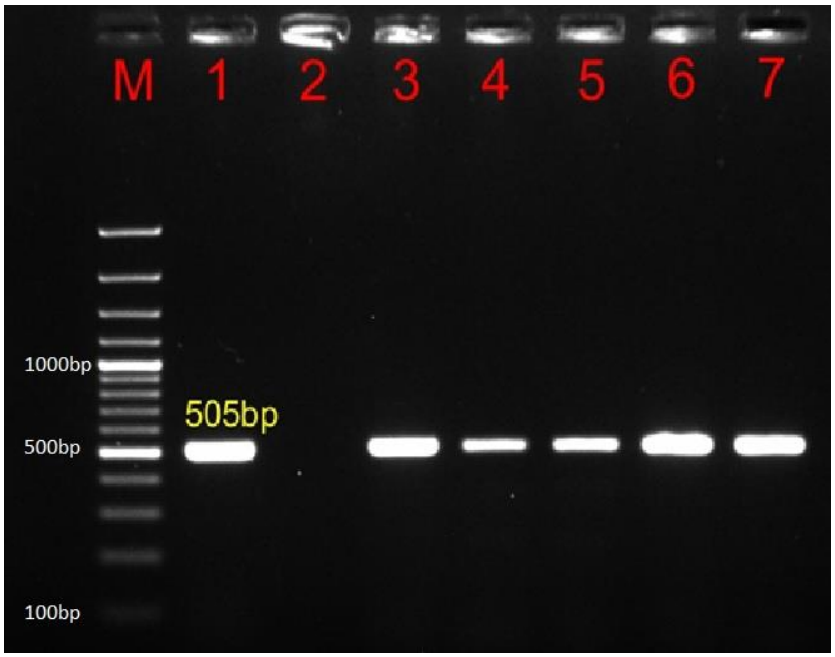


Fig-33: Gel photograph of PCR showing bands for HLAR genes in *Enterococcus* spp.

Lane M	Molecular weight marker (100-3000bp)
Lane 1	Known positive standard of *E. faecalis* showing gene *aac(6´)Ie-aph(2˝)Ia* (505bp)
Lane 2	Negative control
Lane 3	*E. faecium* showing gene *aac(6´)Ie-aph(2˝)Ia* isolated from pork sample
Lane 4	*E. gallinarum* showing gene *aac(6´)Ie-aph(2˝)Ia* isolated from quail sample
Lane 5	*E. casseliflavus* showing gene *aac(6´)Ie-aph(2˝)Ia* isolated from chicken sample
Lane 6&7	*E. faecalis* showing gene *aac(6´)Ie-aph(2˝)Ia* isolated from human urine samples

Table-36: Distribution of β- lactamase genotype (*blaZ*) among different *Enterococcus* spp. isolated from different sources

Samples (No. of *blaZ* positive samples)	*E. faecalis*	*E. faecium*	*E. gallinarum*	*E. casseliflavus*
FOODS OF ANIMAL ORIGIN				
Chicken (34)	15 (44.11%)	11 (32.35%)	3 (8.82%)	5 (14.70%)
Quail (3)	0	1 (33.33%)	2 (66.66%)	0
Mutton (11)	1 (9.09%)	7 (63.63%)	0	3 (27.27%)
Pork (11)	9 (81.81%)	2 (18.18%)	-	0
Fish (14)	1 (7.14%)	9 (64.28%)	4 (28.57%)	0
Carabeef (8)	0	8 (100%)	0	0
Milk (1)	0	1 (100%)	0	0
TOTAL (82)	**26 (31.70%)**	**39 (47.56%)**	**9 (10.97%)**	**8 (9.75%)**
ANIMAL FAECAL SWABS				
Water (1)	0	1 (100%)	0	0
Chicken cloacal swabs (23)	1 (4.34%)	5 (21.73%)	5 (21.73%)	0
Sheep rectal swabs (23)	5 (21.73%)	2 (8.69%)	2 (8.69%)	0
Buffalo rectal swabs (16)	6 (37.5%)	0	0	0
Pig rectal swabs (11)	0	0	1 (9.09%)	0
TOTAL (27)	**12 (44.44%)**	**7 (25.92%)**	**8 (29.62%)**	**0**
HUMAN SAMPLES				
Human Stool samples (7)	2 (28.57%)	5 (71.42%)	0	0
Human diarrhoeic stool samples (3)	0	3 (100%)	0	0
Human urine samples (4)	0	4 (100%)	0	0
TOTAL (14)	**2 (14.28)**	**7 (25.92)**	**8 (29.62)**	**0**
ENVIRONMENTAL SAMPLES				
Water (1)	0	1 (100%)	0	0
MISCELLANEOUS				
Uterine discharges (3)	0	3 (100%)	0	0
Total (127)	**40 (31.49%)**	**62 (48.81%)**	**17 (13.38%)**	**8 (6.29%)**

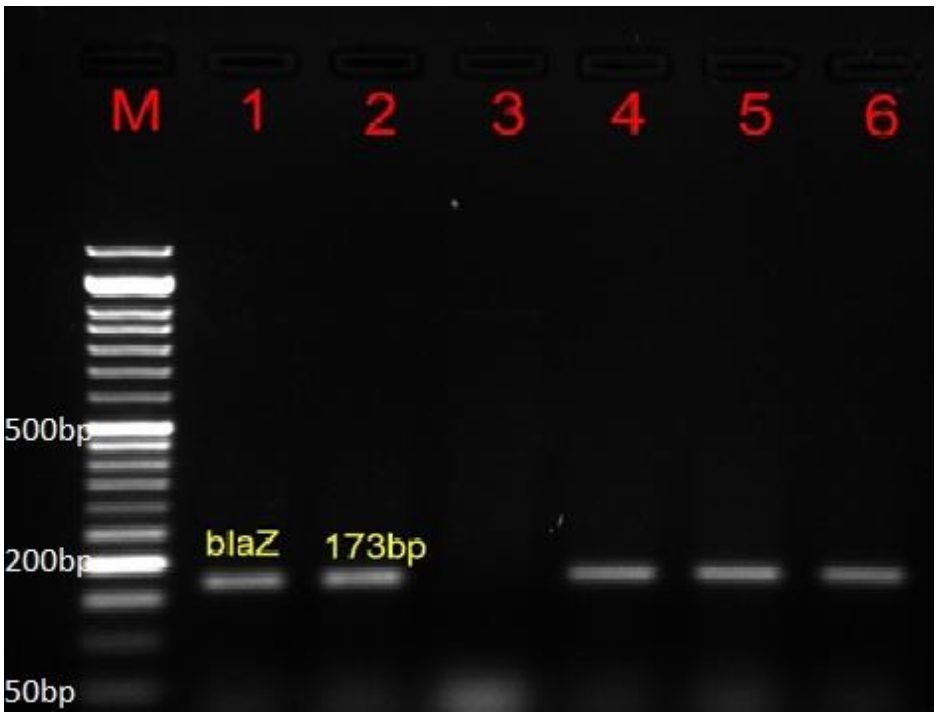


Fig-34: Gel photograph of PCR showing specific band for β-lactamase (*blaZ*) gene in *Enterococcus* spp.

Lane M Molecular weight marker (50-1500bp)

Lane 1 Positive control of MRSA strain showing *blaZ* gene (173bp)

Lane 2 Positive control of *E. faecalis* MTCC439 strain showing gene *blaZ* gene (173bp)

Lane 3 Negative control

Lane 4 *E. faecalis* showing *blaZ* gene isolated from chicken sample

Lane 5 *E. faecium* showing *blaZ* gene isolated from mutton sample

Lane 6 *E. casseliflavus* showing *blaZ* gene isolated from pork sample

4.7.3 Molecular detection of VR genes in *Enterococcus* spp.

A total of 608 *Enterococcus* isolates of different species isolated from different sources were subjected for detection of vancomycin resistance both phenotypically and genotypically using m-PCR assays for detection of 4 major Vancomycin resistant markers like *VanA*, *VanB*, *VanC1* and *VanC2* (Table-37 and 38 and Fig-35) because *vanC* mediated low level resistance is the intrinsic property of *E. gallinarum* and *E. casseliflavus* (Billot-Klein et al., 1994). So all the *vanC* gene carrying genotypes not show phenotypic resistance to vancomycin.

Out of 608 *Enterococcus* isolates, 117 (19.24%) isolates showed resistance to vancomycin by disc diffusion (59 *E. faecalis*, 26 *E. faecium*, 16 *E. gallinarum* and 16 *E. casseliflavus*) and genotypically 125 (20.55%) were found to be VRE. Of 125 VRE positive genotypes, 21 were *E. faecalis* (3 *vanB*, 14 *vanC1* and 4 *vanC2*), 15 *E. faecium* (11 *vanB* and 4 *vanC2*), 58 *E. gallinarum* (52 *vanC1* and 6 *vanC2*) and 31 *E. casseliflavus* (3 *vanC1* and 28 *vanC2*) isolates. None of the isolates showed *vanA* gene. Out of 125 genotypically positive VRE isolates, the *vanB*, *vanC1* and *vanC2* were detected in 14 (11.20%), 69 (55.20%) and 42 (33.60%) VR *Enterococcus* isolates, respectively.

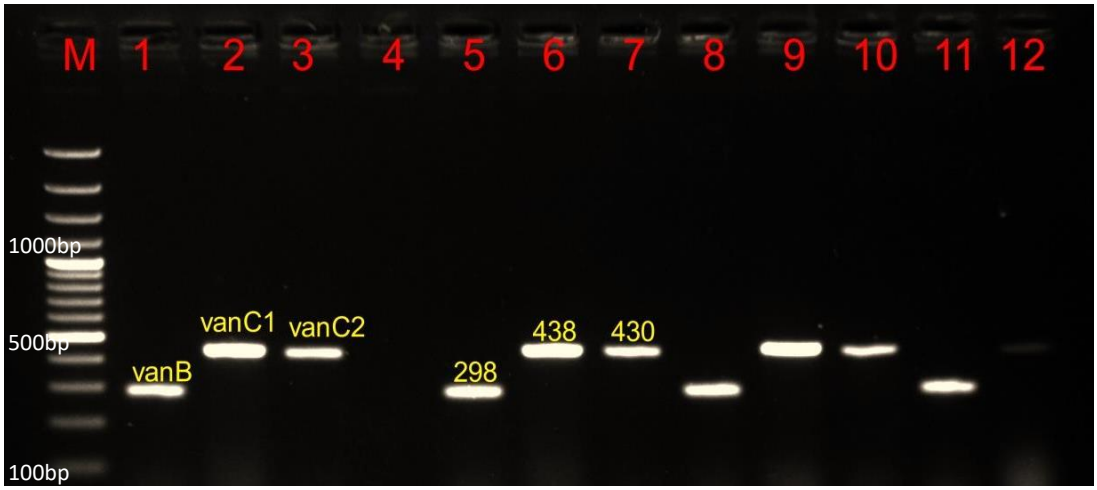


Fig-35: Gel photograph of PCR showing VR specific bands in *Enterococcus* spp.

Lane M Molecular weight marker (100-3000bp)

Lane 1 Known positive standard of *E. faecium* showing gene *vanB* (298bp)

Lane 2 Positive control of *E. gallinarum* MTCC7049 showing gene *vanC1* (438bp)

Lane 3 Known positive standard of *E. casseliflavus* showing gene *vanC2* (430bp)

Lane 4 Negative control

Lane 5 *E. faecium* showing gene *vanB* isolated from water sample (298bp)

Lane 6 *E. gallinarum* showing gene *vanC1* isolated from chicken sample (438bp)

Lane 7 *E. casseliflavus* showing gene *vanC2* isolated from chicken sample (430bp)

Lane 8 *E. faecalis* showing gene *vanB* isolated from human diarrhoeic sample (298bp)

Lane 9 *E. faecalis* showing gene *vanC1* from pork sample (438bp)

Lane 10 *E. gallinarum* showing gene *vanC2* from chicken cloacal swab (430bp)

Lane 11 *E. faecium* showing *vanB* isolated from pork sample (298bp)

Lane 12 *E. casseliflavus* showing *vanC2* isolated from fish sample (430bp)

Table-37: Vancomycin resistant markers among different *Enterococcus* spp. isolated from different sources

Source (No. of VRE isolates)	*E. faecalis*			*E. faecium*			*E. gallinarum*			*E. casseliflavus*		
	VanB	*VanC1*	*VanC2*	*VanB*	*VanC1*	*VanC2*	*VanB*	*VanC1*	*VanC2*	*VanB*	*VanC1*	*VanC2*
FOODS OF ANIMAL ORIGIN												
Chicken (43)	0	5 (11.62%)	2 (4.65%)	5 (11.62%)	0	0	0	13 (30.23%)	5 (11.62%)	0	3 (6.97%)	10 (23.25%)
Quail (13)	0	0	0	0	0	0	0	7 (53.84%)	0	0	0	6 (46.15)
Mutton (13)	0	0	2 (15.38%)	1 (7.69%)	0	0	0	5 (38.46%)	0	0	0	5 (38.46)
Pork (9)	0	6 (66.66%)	0	2 (22.22%)	0	0	0	1 (11.11%)	0	0	0	0
Fish (8)	0	2 (25.00%)	0	0	0	4 (50.00%)	0	0	0	0	0	2 (25.00%)
Carabeef (6)	0	0	0	0	0	0	0	6 (100%)	0	0	0	0
Milk (10)	0	0	0	0	0	0	0	9 (90.00%)	0	0	0	1 (10.00%)
ANIMAL FAECAL SWABS												
Chicken cloacal swabs (5)	0	1 (20.00%)	0	0	0	0	0	2 (40.00%)	1 (20.00%)	0	0	1 (20.00%)
Sheep rectal swabs (2)	0	0	0	0	0	0	0	0	0	0	0	2 (100%)
Buffalo rectal swabs (2)	0	0	0	0	0	0	0	2 (100%)	0	0	0	0
Pig rectal swabs (1)	0	0	0	0	0	0	0	1 (100%)	0	0	0	0
HUMAN SAMPLES												
Human Stool samples (2)	0	0	0	0	0	0	0	2 (100%)	0	0	0	0
Human diarrhoeic stool samples (5)	3 (60.00%)	0	0	0	0	0	0	2 (40.00%)	0	0	0	0
Human urine samples (0)	0	0	0	0	0	0	0	0	0	0	0	0
ENVIRONMENTAL SAMPLES												
Water (6)	0	0	0	3 (50.00%)	0	0	0	2 (33.33%)	0	0	0	1 (16.66%)
MISCELLANEOUS												
Uterine discharges (0)	0	0	0	0	0	0	0	0	0	0	0	0
Total (125)	**3 (2.40%)**	**14 (11.20%)**	**4 (3.20%)**	**11 (8.80%)**	**0**	**4 (3.20%)**	**0**	**52 (41.60%)**	**6 (4.80%)**	**0**	**3 (2.40%)**	**28 (22.40%)**

Table-38: Distribution of VR, HLAR and β- lactamase resistance genes in different *Enterococcus* spp.

Antimicrobial resistance genes	*E. faecalis* (n= 278)	*E. faecium* (n= 179)	*E. gallinarum* (n= 103)	*E. casseliflavus* (n= 48)
HLAR (*aac(6´)Ie-aph(2˝)Ia*)	95 (34.17%)	44 (24.58)	30 (29.12%)	10 (20.83%)
blaZ	40 (14.38%)	62 (34.63%)	17 (16.50%)	8 (16.66%)
VRE (*vanB, vanC1* and *vanC2*)	21 (7.55%)	15 (8.37%)	58 (56.31%)	31 (64.58%)

4.7.4 Mutidrug Resistance in VRE spp.

Organisms showing resistance towards three or more classes are considered as MDR organisms. MDR can also be evaluated by Multiple Antibiotic Resistance (MAR) index (Kruperman, 1983). If MAR index is greater than **0.2**, it implies that those strains originated from an environment that is exposed to several antibiotics.

MAR index of single isolate = A/B where,

A - number of antibiotics to which the isolate was resistant

B- number of antibiotics to which the isolate was exposed (n=16)

Out of 125 (21 *E. faecalis*, 15 *E. faecium*, 58 *E. gallinarum* and 31 *E. casseliflavus*) VRE isolates, 114 (19 *E. faecalis*, 15 *E. faecium*, 49 *E. gallinarum* and 31 *E. casseliflavus*) isolates were MDR.

4.7.4.1 MAR indexing of VR *E. faecalis* isolates

MAR index values for VR *E. faecalis* were predominantly found to be above 0.2 except 8 isolates. The average MAR index value was found to be 0.29 (Table-39). Depending on the combination of different resistance antibiotics and MAR index value i.e. >0.2, 21 *E. faecalis* were divided into 19 different MAR index groups.

Table-39: Detection of MAR index among VR *E. faecalis* isolates

Sample ID	MDR index label	No of isolates	Resistance antibiotics	Total no. of antibiotics the isolate was resistant to (A)	MAR index value (A/B)
c1 and hd3	MARF1	2	PB and CAZ	2	0.125
p17	MARF2	1	S and PB	2	0.125
c17	MARF3	1	S, PB and CAZ	3	0.187
m12	MARF4	1	LZ, GEN and PB	3	0.187
p11	MARF5	1	E, GEN and PB	3	0.187
p27	MARF6	1	LZ, PB and CAZ	3	0.187
p50	MARF7	1	S, E and PB	3	0.187
c136	MARF8	1	LZ, C, PB and CAZ	4	0.25
m13	MARF9	1	RIF, VA, PB and CAZ	4	0.25
c9	MARF10	1	S, E, C, P and PB	5	0.3125
c21	MARF11	1	CIP, S, TE, C and PB	5	0.3125
f11	MARF12	1	CIP, S, PI, PB and CAZ	5	0.3125
p31	MARF13	1	CIP, S, C, PB and CAZ	5	0.3125
c39	MARF14	1	CIP, RIF, TE, GEN, PB and CAZ	6	0.375
f22	MARF15	1	S, VA, TEI, C, PB and CAZ	6	0.375
c110	MARF16	1	CIP, E, TEI, GEN, P, AMP and PB	7	0.4375
p53	MARF17	1	CIP, RIF, TE, E, GEN, P and PB	7	0.4375
hd6	MARF18	1	CIP, RIF, NIT, TEI, C, AMP and PB	7	0.4375
hd2 and CC26	MARF19	2	CIP, S, VA, TE, E, P, PB and CAZ	8	0.125

4.7.4.2 MAR indexing of VR *E. faecium* isolates

MAR index values for VR *E. faecium* were predominantly found to be above 0.2 except 2 isolates. The average MAR index value was found to be 0.345 (Table-40). Depending on the combination of different resistance antibiotics and MAR index value i.e. >0.2, 15 *E. faecium* were divided into 13 different MAR index groups.

Table-40: Detection of MAR index value among VR *E. faecium* isolates

Sample ID	MDR index label	No of isolates	Resistance antibiotics	Total no. of antibiotics the isolate was resistant to (A)	MAR index value (A/B)
c76 and p11	MARf1	2	CIP, TE and PB	3	0.1875
c47	MARf2	1	S, LZ, PB and CAZ	4	0.25
c81	MARf3	1	E, GEN, PB and CAZ	4	0.25
f41	MARf4	1	VA, E, P and PB	4	0.25
c84	MARf5	1	CIP, S, TE, PB and CAZ	5	0.3125
p14	MARf6	1	CIP, TE, E, PB and CAZ	5	0.3125
f39	MARf7	1	CIP, S, LZ, C and PB	5	0.3125
c62	MARf8	1	CIP, S, LZ, E, PB and CAZ	6	0.375
w21	MARf9	1	S, LZ, E, C, PB and CAZ	6	0.375
f36	MARf10	1	S, TE, E, AMP, PB and CAZ	6	0.375
w22	MARf11	1	CIP, S, TE, E, GEN, PB and CAZ	7	0.4375
w12 and f14	MARf12	2	CIP, S, LZ, GEN, C, AMP, PB and CAZ	8	0.5
m10	MARf13	1	S, RIF, E, TEI, P, AMP, PI, PB and CAZ	9	0.5625

4.7.4.3 MAR indexing of VR *E. gallinarum* isolates

MAR index values for VR *E. gallinarum* were predominantly found to be above 0.2 except 18 isolates. The average MAR index value was found to be 0.365 (Table-41). Depending on the combination of different resistance antibiotics and MAR index value i.e. >0.2, 58 *E. faecalis* were divided into 50 different MAR index groups.

Table-41: Detection of MAR index among VR *E. gallinarum* isolates

Sample ID	MDR index label	No of isolates	Resistance antibiotics	Total no. of antibiotics the isolate was resistant to (A)	MAR index value (A/B)
h17 and M79	MARG1	2	PB	1	0.0625
c112 and c120	MARG2	2	E and PB	2	0.125
c127	MARG3	1	RIF and PB	2	0.125
CC13 and m26	MARG4	2	S and PB	2	0.125
hd1	MARG5	1	TEI and PB	2	0.125
w18	MARG6	1	RIF and PB	2	0.125
CC8	MARG7	1	E, GEN and PB	3	0.1875
cb11	MARG8	1	S, E and PB	3	0.1875
cb17 and q24	MARG9	2	S, TE and PB	3	0.1875
h20	MARG10	1	PI, PB and CAZ	3	0.1875
M19	MARG11	1	GEN, PB and CAZ	3	0.1875
M39	MARG12	1	S, PB and CAZ	3	0.1875
M4	MARG13	1	TE, GEN and PB	3	0.1875
M77	MARG14	1	VA, TE and PB	3	0.1875
c115	MARG15	1	S, TE, E and PB	4	0.25
c83	MARG16	1	S, TE, GEN and PB	4	0.25
PR6	MARG17	1	S, RIF, E and PB	4	0.25
M43	MARG18	1	CIP, NIT, E and PB	4	0.25
q50	MARG19	1	E, GEN, P and PB	4	0.25
CC20	MARG20	1	S, TE, E, GEN and PB	5	0.3125
cb21	MARG21	1	CIP, RIF, E, PB and CAZ	5	0.3125
hd5	MARG22	1	S, E, GEN, PB and CAZ	5	0.3125
M3	MARG23	1	S, TE, GEN, PB and CAZ	5	0.3125
m5	MARG24	1	CIP, S, TE, E and PB	5	0.3125
m52	MARG25	1	RIF, E, AMP, PB and CAZ	5	0.3125
m21, c3 and c79	MARG26	3	S, TE, E, GEN, PB and CAZ	6	0.375
CC10	MARG27	1	RIF, TE, E, GEN, PB and CAZ	6	0.375
CC19	MARG28	1	E, GEN, C, P, PB and CAZ	6	0.375
c31	MARG29	1	CIP, S, TE, E, GEN and PB	6	0.375
c33	MARG30	1	CIP, TE, E, C, PB and CAZ	6	0.375
cb29	MARG31	1	CIP, S, E, C, PB	6	0.375

			and CAZ		
w13	MARG32	1	S, E, GEN, P, PB and CAZ	6	0.375
CC31 and q46	MARG33	2	CIP, S, TE, E, C, PB and CAZ	7	0.4375
M76	MARG34	1	S, E, GEN, C, P, PB and CAZ	7	0.4375
c159	MARG35	1	CIP, S, TE, E, GEN, C, PB and CAZ	8	0.5
c65	MARG36	1	CIP, S, E, GEN, C, P, PB and CAZ	8	0.5
M77	MARG37	1	CIP, S, LZ, RIF, E, GEN, PB and CAZ	8	0.5
c101	MARG38	1	S, RIF, E, GEN, C, P, AMP, PB and CAZ	9	0.5625
p36	MARG39	1	CIP, S, LZ, RIF, VA, NIT, TE, PB and CAZ	9	0.5625
q27	MARG40	1	NIT, E, GEN, C, P, AMP, PI, PB and CAZ	9	0.5625
cb5	MARG41	1	CIP, S, LZ, RIF, VA, NIT, TE, PI, PB and CAZ	10	0.625
M24	MARG42	1	CIP, S, E, TEI, GEN, C, P, AMP, PB and CAZ	10	0.625
q36	MARG43	1	CIP, S, LZ, RIF, VA, NIT, E, GEN, PB and CAZ	10	0.625
c88	MARG44	1	S, RIF, VA, E, TEI, GEN, P, AMP, PI, PB and CAZ	11	0.6875
m25	MARG45	1	CIP, S, LZ, R, E, GEN, C, P, AMP, PB and CAZ	11	0.6875
q22	MARG46	1	CIP, RIF, E, TEI, GEN, C, P, AMP, PI, PB and CAZ	11	0.6875
q31	MARG47	1	CIP, S, LZ, RIF, VA, NIT, E, GEN, P, PB and CAZ	11	0.6875
BR9	MARG48	1	CIP, S, LZ, RIF, E, TEI, GEN, C, P, AMP, PI, PB and CAZ	13	0.8125
c153	MARG49	1	LZ, RIF, VA, NIT, TE, E, TEI, GEN, C, P, AMP, PB and CAZ	13	0.8125
c99	MARG50	1	CIP, S, LZ, RIF, TE, E, R, GEN, C, P, AMP, PI, PB and CAZ	14	0.875

4.7.4.4 MAR indexing of VR *E. casseliflavus* isolates

MAR index values for VR *E. casseliflavus* were predominantly found to be above 0.2 except 2 isolates. The average MAR index value was found to be 0.345 (Table-42). Depending on the combination of different resistance antibiotics and MAR index value i.e. >0.2, 31 *E. casseliflavus* were divided into 30 different MAR index groups.

Table-42: Detection of MAR index value among VR *E. casseliflavus* isolates

Sample ID	MDR index label	No of isolates (A)	Resistance antibiotics	Total no. of antibiotics the isolate was resistant to (A)	MAR index value (A/B)
c142	MARC1	1	C, PB and CAZ	3	0.1875
c25	MARC2	1	VA, PB and CAZ	3	0.1875
m6	MARC3	1	CIP, RIF, AMP and PB	4	0.25
c11	MARC4	1	CIP, TE, GEN and PB	4	0.25
c13	MARC5	1	CIP, S, LZ, RIF and PB	5	0.3125
f35	MARC6	1	CIP, S, E, GEN and PB	5	0.3125
M8	MARC7	1	LZ, NIT, PI, PB and CAZ	5	0.3125
w10	MARC8	1	CIP, RIF, C, AMP and PB	5	0.3125
SR8	MARC9	1	CIP, S, E, C and PB	5	0.3125
q2	MARC10	1	S, RIF, E, PB and CAZ	5	0.3125
q15	MARC11	1	CIP, VA, TE, PB and CAZ	5	0.3125
q44	MARC12	1	CIP, S, TE, E, P and PB	6	0.375
c119	MARC13	1	CIP, S, TE, E, PB and CAZ	6	0.375
c109	MARC14	1	CIP, LZ, NIT, C, AMP and PB	6	0.375
c94	MARC15	1	S, RIF, E, P, PB and CAZ	6	0.375
c32	MARC16	1	CIP, S, E, C, AMP and PB	6	0.375
c66	MARC17	1	LZ, RIF, TE, E, PB and CAZ	6	0.375
q49	MARC18	1	S, RIF, GEN, AMP, PB and CAZ	6	0.375
m30	MARC19	1	LZ, NIT, TE, GEN, P, PB and	7	0.4375

			CAZ		
c27	MARC20	1	CIP, S, LZ, E, GEN, PB and CAZ	7	0.4375
c93	MARC21	1	S, RIF, VA, GEN, P, AMP and PB	7	0.4375
c122	MARC22	1	CIP, S, LZ, C, AMP, PB and CAZ	7	0.4375
c56	MARC23	1	CIP, RIF, NIT, TE, GEN, PB and CAZ	7	0.4375
f13 and q5	MARC24	2	CIP, S, RIF, VA, E, PB and CAZ	7	0.4375
SR6	MARC25	1	S, RIF, TE, TEI, GEN, P, PI and PB	8	0.5
CC17	MARC26	1	S, RIF, VA, TE, E, C, PB and CAZ	8	0.5
c139	MARC27	1	TE, E, TEI, GEN, P, PI, PB and CAZ	8	0.5
m39	MARC28	1	CIP, S, RIF, NIT, E, TEI, P, PB and CAZ	9	0.5625
q54	MARC29	1	CIP, LZ, TE, E, TEI, GEN, P, AMP, PI and PB	10	0.625
c146	MARC30	1	CIP, S, LZ, RIF, NIT, E, C, P, PI, PB ND CAZ	11	0.6875

4.8 GENETIC DIVERSITY OF VR *ENTEROCOCCUS* SPECIES

A total of 125 VR *Enterococcus* isolates (21 *E. faecalis*, 15 *E. faecium*, 58 *E. gallinarum* and 31 *E. casseliflavus*) including two MTCC strains (*E. faecalis* MTCC439 and *E. gallinarum* MTCC7049) isolates were characterized further by two typing methods (ERIC and REP-PCR) in triplicate, in order to differentiate them based on genetic diversity and to assess the intra-specific diversity. ERIC-PCR and REP-PCR fingerprinting profiles were visualized under UV transilluminator, photographed and compared for similarity by visual inspection of the band profiles as well as by using image lab software (BIO-RAD). Both ERIC and REP-PCR sequences were found to be present in 124 VR *Enterococcus* isolates (one *E. gallinarum* isolate was undifferentiated). Patterns with at least one different band were considered as different genotypes. In other words, isolates that had patterns showing the same number of bands with same size of corresponding bands were considered as indistinguishable or same genotype. Dendrograms were constructed for the VR *Enterococcus* spp. isolates, from the binary scores obtained from ERIC and REP-PCR fingerprint data.

4.8.1 Genetic diversity of VR *E. faecalis* isolates

ERIC-PCR typing revealed 4-9 fragments per isolate, ranging in size from ~100 bp to ~2000 bp, whereas REP-PCR typing revealed 3-12 fragments resolved per isolate, ranging in size from ~100 bp to ~2000 bp. Of the 21 VR *E. faecalis* analyzed, 19 ERIC-PCR patterns and 21 REP patterns were obtained. The binary score demonstrating the variety of 19 ERIC (E1-E19) and 21 REP PCR genotypes (R1-R21) were given in Table- 43 and 44, respectively. Four *E. faecalis* isolates (E18 and E19) that had identical ERIC-PCR band pattern were distinguishable in REP-PCR pattern (R18, R19, R20 and R21). Dendrograms were constructed based on ERIC and REP-PCR profiles (Fig-36 to 39) using dollop program of phylip 3.6 version.

Dendrogram analysis of ERIC-PCR profiles discriminated VR *E. faecalis* isolates into seven major clusters. Cluster I comprised three isolates (c1, c9 and c17) of chicken samples where isolate c1 clustered separately from that of other two isolates. In cluster II, *E. faecalis* MTCC439 was closely clustered with isolate of mutton origin (m13) showing 90% similarity cut off. Clusters III and IV were having two sub clusters each with two isolates in each sub cluster. Cluster III comprised of four isolates of pork origin (p27, p31, p11 and p50). Cluster V, VI and VII were having two isolates each. Cluster VI comprised of human diarrhoeic isolate (hd3) and chicken cloacal swab isolate (CC26) with same ERIC-PCR band pattern. Cluster VII consisted of two isolates of human diarrhoeic origin (hd2 and hd6) having similar ERIC band pattern. Three isolates (c21, f11 and c136) were found to be unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates.

Dendrogram analysis of REP-PCR profiles discriminated VR *E. faecalis* isolates into a four major clusters. Cluster I was again divided into two sub clusters having two isolates each. Cluster II has three isolates (hd2, hd6 and p53) where p53 isolate was distantly related. Cluster III has two isolates (m13 and c9) whereas Clusters IV and V have four isolates each. In cluster IV, isolate from chicken (c21) was distantly separated from other three isolates (p31, c136 and p50). In cluster V, isolate from pork (p11) was separated from other three isolates (m12, c17 and CC26). Four isolates (c1, c39, hd3 and p27) and *E. faecalis* MTCC439 were found to be unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates.

4.8.2 Genetic diversity of VR *E. faecium* isolates

ERIC-PCR typing revealed 3-11 fragments per isolate, ranging in size from ~100 bp to ~2000 bp, whereas REP-PCR typing revealed 3-14 fragments resolved per isolate, ranging in size from ~100 bp to ~2000 bp. Of the 15 VR *E. faecium* analyzed,

Table-43 Scoring of VR *E. faecalis* ERIC-PCR genotypes

Band pattern	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14	E15	E16	E17	E18	E18	E19	E19	MTCC
Sample id	c1	p17	c17	m12	p11	p27	p50	c136	m13	c9	c21	f11	p31	c39	f22	c110	p53	hd6	hd2	hd3	CC26	
Band 1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	1	1	1
Band 2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 4	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 5	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
Band 7	1	1	1	1	1	0	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1
Band 8	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 9	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 10	0	1	1	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0
Band 11	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 12	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Band 13	1	0	0	0	0	0	1	1	0	1	0	0	0	0	1	1	0	0	0	0	0	1
Band 14	0	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
Band 15	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Band 16	0	0	1	1	0	0	0	0	0	1	0	1	1	0	0	0	0	1	1	1	1	0
Band 17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
Band 18	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0		0
Band 19	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 20	0	0	0	0	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1
Band 21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 22	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0
Band 23	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Band 24	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0
Band 25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 26	0	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	0	1	1	1	1	1
Band 27	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 28	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 29	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 30	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 32	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0
Band 33	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
TOTAL	**5**	**9**	**5**	**9**	**5**	**4**	**6**	**4**	**4**	**6**	**4**	**3**	**4**	**4**	**7**	**6**	**8**	**5**	**5**	**5**	**5**	**9**

Isolates of chicken: E1, E3, E8, E10, E11, E14 and E16. **Isolates of pork:** E2, E5, E6, E7, E13 and E17. **Isolates of mutton:** E4 and E9. **Isolates of fish:** E12 and E15. **Isolates of chicken cloacal swabs:** E21. **Isolates of human diarrhoea:** E18, E18 and E19.

Indistinguishable ERIC-PCR patterns E18 and E19 which has distinguishable REP-PCR patterns.

Number of VR *E. faecalis* isolates : 21
Number of VR *E. faecalis* ERIC-PCR genotypes: 19

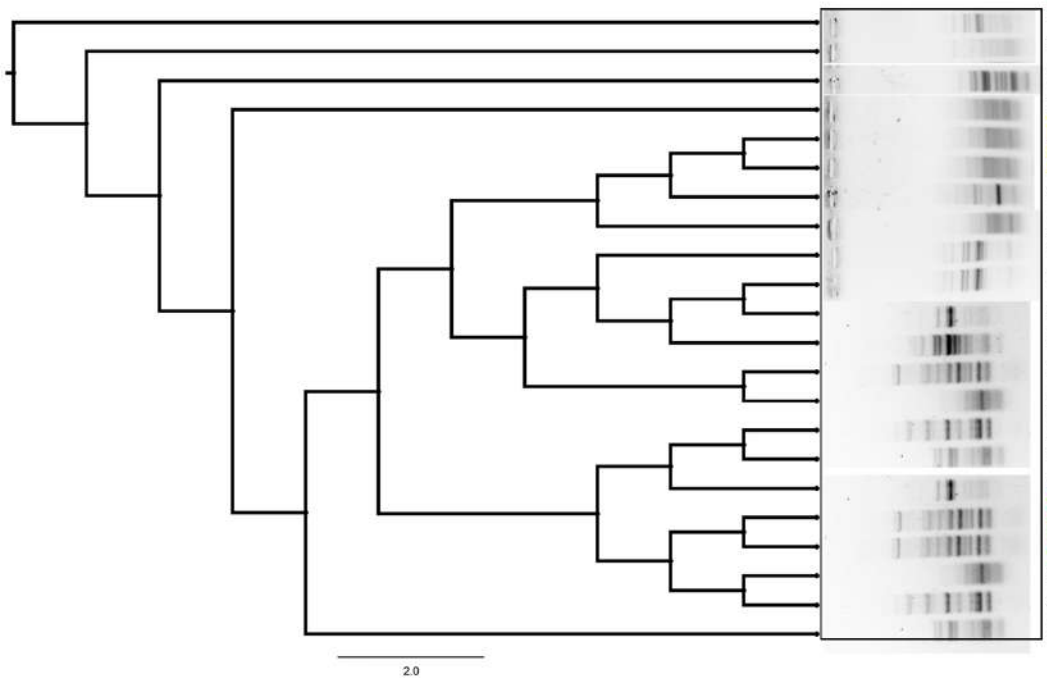
Table 44: Scoring of VR *E. faecalis* REP-PCR genotypes

Band pattern	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15	R16	R17	R18	R19	R20	R21	MTCC
Sample id	c1	p17	c17	m12	p11	p27	p50	c136	m13	c9	c21	f11	p31	c39	f22	c110	p53	hd6	hd2	hd3	CC26	MTCC
Band 1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Band 2	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 3	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 4	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
Band 5	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Band 6	0	0	1	0	1	1	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0
Band 7	0	0	0	0	0	1	1	1	0	1	0	1	0	0	0	1	0	0	0	1	0	0
Band 8	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Band 9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 10	0	0	0	0	0	0	1	1	0	1	0	1	1	1	1	1	0	0	0	1	0	0
Band 11	0	0	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0
Band 12	0	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0
Band 13	1	1	0	0	0	0	0	0	1	0	0	1	1	0	1	1	1	1	0	0	0	0
Band 14	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
Band 15	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	1
Band 16	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
Band 17	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
Band 18	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
Band 19	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
Band 20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Band 21	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0	0	1
Band 22	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1
Band 23	0	0	0	0	0	0	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0	1

Band 24	0	0	0	0	0	0	1	0	1	1	0	0	1	0	0	1	0	0	0	0	0
Band 25	0	0	0	1	1	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0
Band 26	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
Band 27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0
Band 28	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Band 30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0
Band 31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
Band 32	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 33	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Band 34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Band 35	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0
Band 36	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 37	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0
Band 38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Band 39	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0
Band 40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Band 41	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1
Band 42	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Band 43	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Band 44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Band 45	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
TOTAL	4	5	6	8	9	6	7	5	11	12	6	6	7	6	9	9	5	8	8	11	3	9

Isolates of chicken: R1, R3, R8, R10, R11, R14 and R16. **Isolates of pork:** R2, R5, R6, R7, R13 and R17. **Isolates of mutton:** R4 and R9. **Isolates of human diarrhoea:** R18, R19 and R20. **Isolates of fish:** R12 and R15. **Isolates of chicken cloacal swabs:** R21.

Number of VR *E. faecalis* isolates : 21
Number of VR *E. faecalis* REP-PCR genotypes: 21



Sample ID	Biological origin	Place	ERIC Pattern
c21	chicken	Gannavaram	E11
f11	fish	Gannavaram	E12
c110	chicken	LPT Gannavaram	E16
f22	fish	Kesarapalli	E15
hd2	Human diarrhoea	Vijayawada	E18
hd6	Human diarrhoea	Siddhartha	E18
c136	chicken	Gudivada	E8
p53	Pork	LPT Gannavaram	E17
m12	mutton	Kankipadu	E4
c39	chicken	Gudivada	E14
p17	Pork	LPT Gannavaram	E2
p50	Pork	LPT Gannavaram	E7
p11	Pork	LPT Gannavaram	E5
p31	Pork	LPT Gannavaram	E13
p27	Pork	LPT Gannavaram	E6
m13	mutton	Gannavaram	E9
MTCC	Standard MTCC439		
hd3	Human diarrhoea	Siddhartha	E19
CC26	chicken cloacal swab	LFC Gannavaram	E19
c17	chicken	Gannavaram	E3
c9	chicken	Gannavaram	E10
c1	chicken	Gannavaram	E1

Indistinguishable ERIC-PCR patterns E18 and E19 which had distinguishable REP-PCR patterns

Fig-36 Dendrogram analysis of ERIC-PCR fingerprints of VR *E. faecalis* isolates from different sources. The dendrogram was generated by the "Branch-and-bound" method using dollop program of PHYLIP 3.6 version

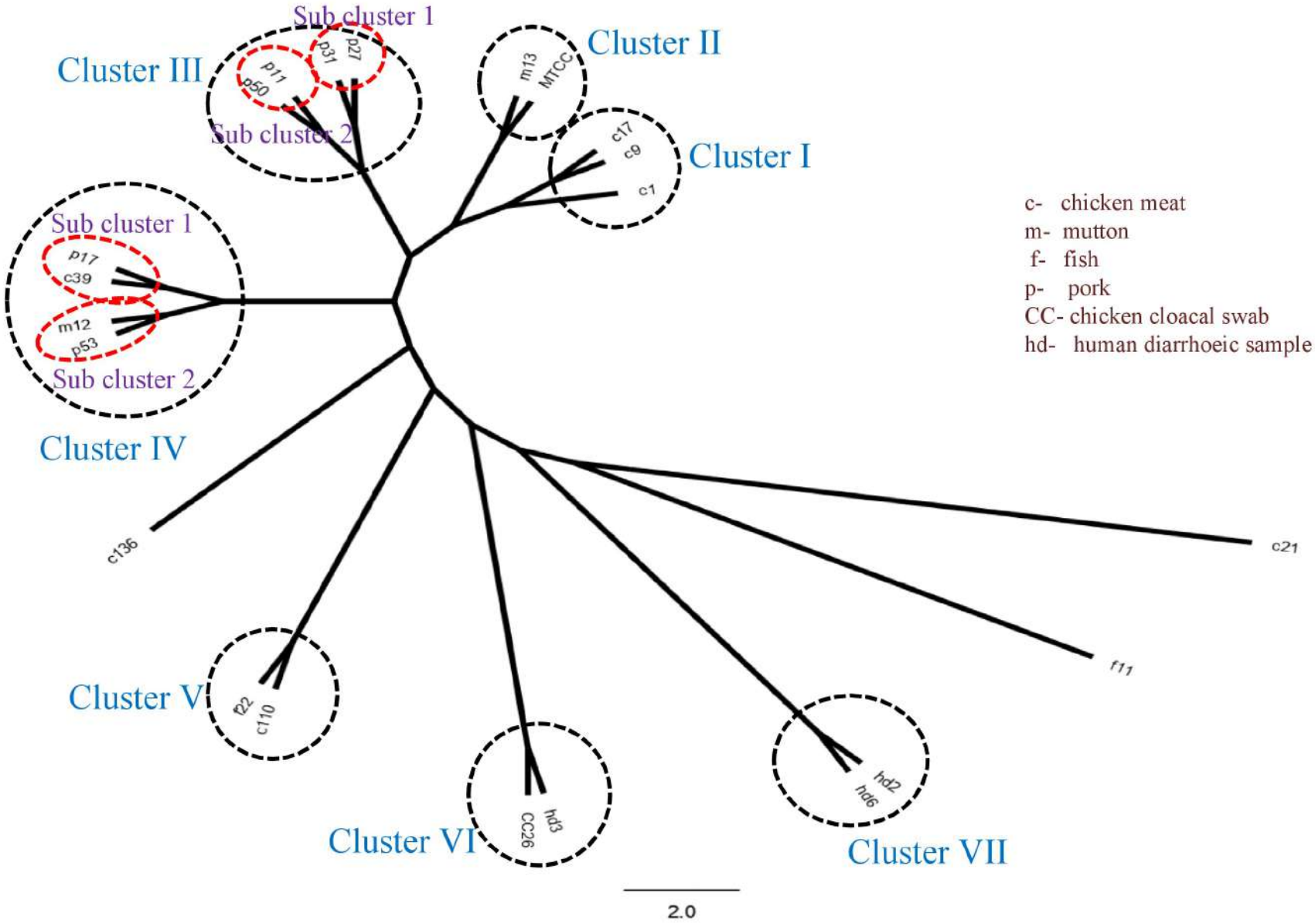
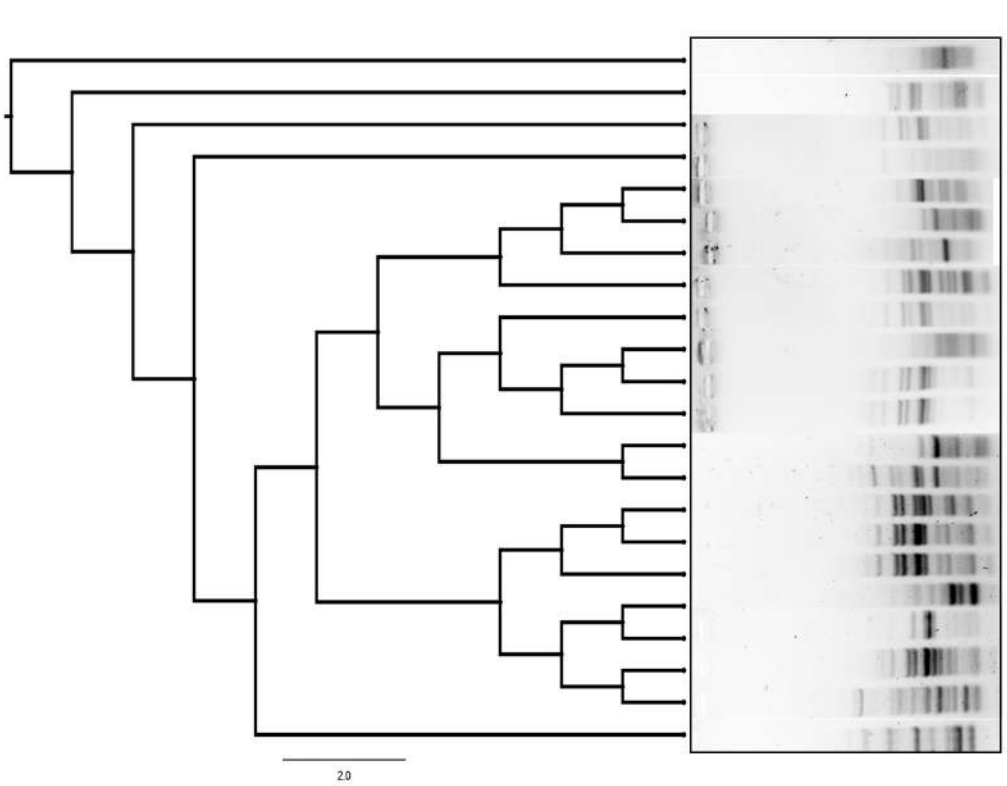


Fig-37: Cluster analysis of ERIC-PCR fingerprints of VR *E. faecalis* from different sources.
An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm)



Sample ID	Biological origin	Place	REP Pattern
c39	chicken	Gudivada	R14
hd3	Human diarrhoea	Siddhartha	R20
p27	Pork	LPT Gannavaram	R6
MTCC	Standard MTCC439		
CC26	chicken cloacal swab	LFC Gannavaram	R21
c17	chicken	Gannavaram	R3
m12	mutton	Kankipadu	R4
p11	Pork	LPT Gannavaram	R5
c21	chicken	Gannavaram	R11
p50	Pork	LPT Gannavaram	R7
c136	chicken	Gudivada	R8
p31	Pork	LPT Gannavaram	R13
c9	chicken	Gannavaram	R10
m13	mutton	Gannavaram	R9
hd2	Human diarrhoea	Vijayawada	R19
hd6	Human diarrhoea	Siddhartha	R18
p53	Pork	LPT Gannavaram	R17
c110	chicken	LPT Gannavaram	R16
f22	fish	Kesarapalli	R15
f11	fish	Gannavaram	R12
p17	Pork	LPT Gannavaram	R2
c1	chicken	Gannavaram	R1

Fig-38 Dendrogram analysis of REP-PCR fingerprints of VR *E. faecalis* isolates from different sources. The dendrogram was generated by the "Branch-and-bound" method using dollop program of PHYLIP 3.6 version

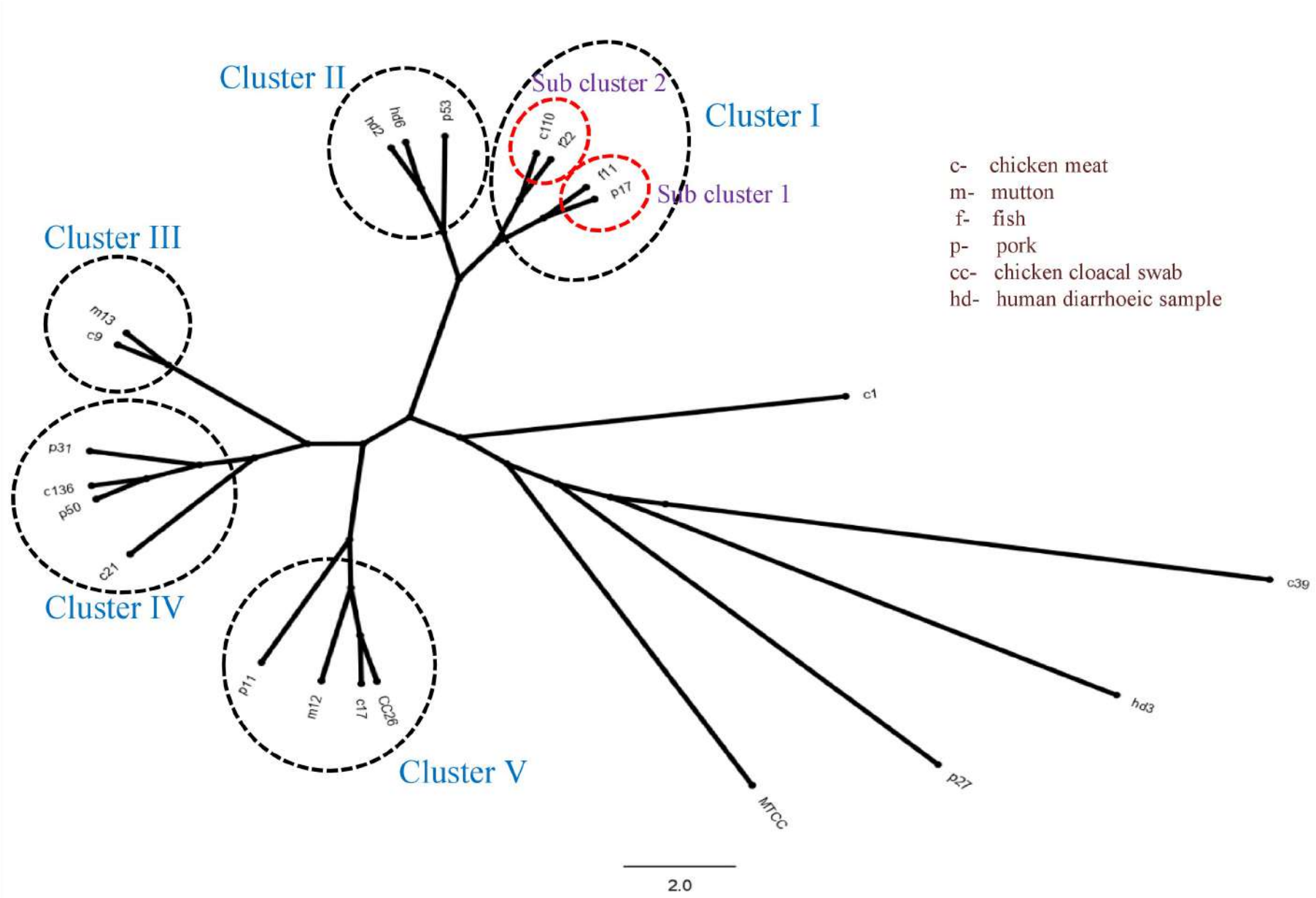


Fig-39: Cluster analysis of REP-PCR fingerprints of VR *E. faecalis* isolates from different sources.
An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm)

15 ERIC-PCR patterns and 15 REP patterns were obtained. The binary score demonstrating the variety of 15 ERIC (E1-E15) and REP PCR genotypes (R1-R15) were given in Table- 45 and 46, respectively. Dendrograms were constructed based on ERIC and REP-PCR profiles (Fig-40 to 43) using dollop program of phylip 3.6 version.

Dendrogram analysis of ERIC-PCR profiles discriminated VR *E. faecium* isolates into four major clusters. Cluster I again divided into two sub clusters, each sub cluster having two isolates. Cluster I (c76, p14, m10 and f39) and cluster II (p11, f14, c47 and c81) comprised isolates of meat origin. Within the cluster II, isolates p11 and c81 were clustered separately from that of other two isolates (f14 and c47). Cluster III comprised of three isolates of which two were of fish origin (f41 and f36) and one isolate was recovered from water sample (w22). In cluster IV, water isolate (w12) was closely associated with chicken meat isolate (c84). Two isolates (w21 and c62) were found to be unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates.

Dendrogram analysis of REP-PCR profiles discriminated VR *E. faecium* isolates into four major clusters. Cluster I and II have two isolates each (c76 and p11; c84 and f39, respectively). Within the cluster III, isolates w12 and m10 were separated from that of other two isolates (f36 and w22). In cluster IV, w21 and c62 were clustered separately from other two isolates (c81 and f14). Three isolates (p14, f41 and c47) were found to be unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates.

4.8.3 Genetic diversity of VR *E. gallinarum* isolates

ERIC-PCR typing revealed 1-11 fragments per isolate, ranging in size from ~100 bp to ~2000 bp, whereas REP-PCR typing revealed 1-11 fragments per isolate, ranging in size from ~100 bp to ~2000 bp. One isolate of *E. gallinarum* did not yield

Table-45 Scoring of VR *E. faecium* ERIC-PCR genotypes

Band pattern	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14	E15
Sample id	c76	p11	c47	c81	f41	c84	p14	f39	c62	w21	f36	w22	w12	f14	m10
Band 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 2	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1
Band 3	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Band 4	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1
Band 5	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1
Band 6	1	1	1	1	1	0	1	0	0	0	0	0	0	1	1
Band 7	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
Band 8	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
Band 9	0	1	1	0	0	0	0	1	0	1	0	0	0	0	0
Band 10	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0
Band 11	0	1	0	1	0	0	0	0	0	0	0	0	0	1	1
Band 12	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0
Band 13	0	1	0	1	1	0	1	0	0	0	0	0	0	0	1
Band 14	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Band 15	0	0	1	1	0	0	0	0	0	1	0	1	1	0	1
Band 16	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 17	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
Band 18	0	0	0	0	1	1	1	0	0	0	1	1	1	1	0
Band 19	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
Band 20	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Band 21	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0
Band 22	0	0	0	0	1	1	1	0	0	0	1	1	1	0	0
Band 23	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 24	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
Band 25	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 26	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1
Band 27	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 28	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
TOTAL	5	9	5	9	5	4	6	4	4	6	4	3	4	3	11

Isolates of chicken: E1, E3, E4, E6 and E9. **Isolates of fish:** E5, E8, E11 and E14. **Isolates of water:** E10, E12 and E13. **Isolates of pork:** E2 and E7. **Isolates of mutton:** E15.

Number of VR *E. faecium* isolates : 15
Number of VR *E. faecium* ERIC-PCR genotypes: 15

Table-46 Scoring of VR *E. faecium* REP-PCR genotypes

Band pattern	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15
Sample id	c76	p11	c47	c81	f41	c84	p14	f39	c62	w21	f36	w22	w12	f14	m10
Band 1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Band 2	0	0	0	1	1	0	1	1	1	1	0	0	0	0	0
Band 3	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
Band 4	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Band 5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Band 6	0	0	0	0	1	1	1	1	1	1	1	1	0	1	1
Band 7	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 8	1	1	1	0	1	0	0	0	1	1	0	0	1	0	0
Band 9	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Band 10	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Band 11	0	0	0	1	0	0	0	0	0	1	0	0	1	1	1
Band 12	0	0	0	1	0	0	0	0	1	1	0	0	0	1	0
Band 13	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
Band 14	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Band 15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Band 16	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1
Band 17	1	0	0	0	0	0	1	0	1	0	1	1	0	0	0
Band 18	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Band 19	0	0	0	0	0	1	0	1	1	1	0	0	0	0	0
Band 20	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Band 21	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Band 22	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0
Band 23	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
Band 24	0	0	0	0	1	1	1	1	0	0	1	1	1	0	1
Band 25	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 26	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Band 27	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 28	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1
Band 29	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
Band 30	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Band 31	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Band 32	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Band 33	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Band 34	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
Band 35	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 36	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
Band 37	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 38	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Band 39	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0
Band 40	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0
TOTAL	3	3	4	5	7	9	14	8	10	6	5	6	9	4	7

Isolates of chicken: R1, R3, R4, R6 and R9. **Isolates of fish:** R5, R8, R11 and R14. **Isolates of water:** R10, R12 and R13. **Isolates of pork:** R2 and R7. **Isolates of mutton:** R15.

Number of VR *E. faecium* isolates : 15
Number of VR *E. faecium* REP-PCR genotypes: 15

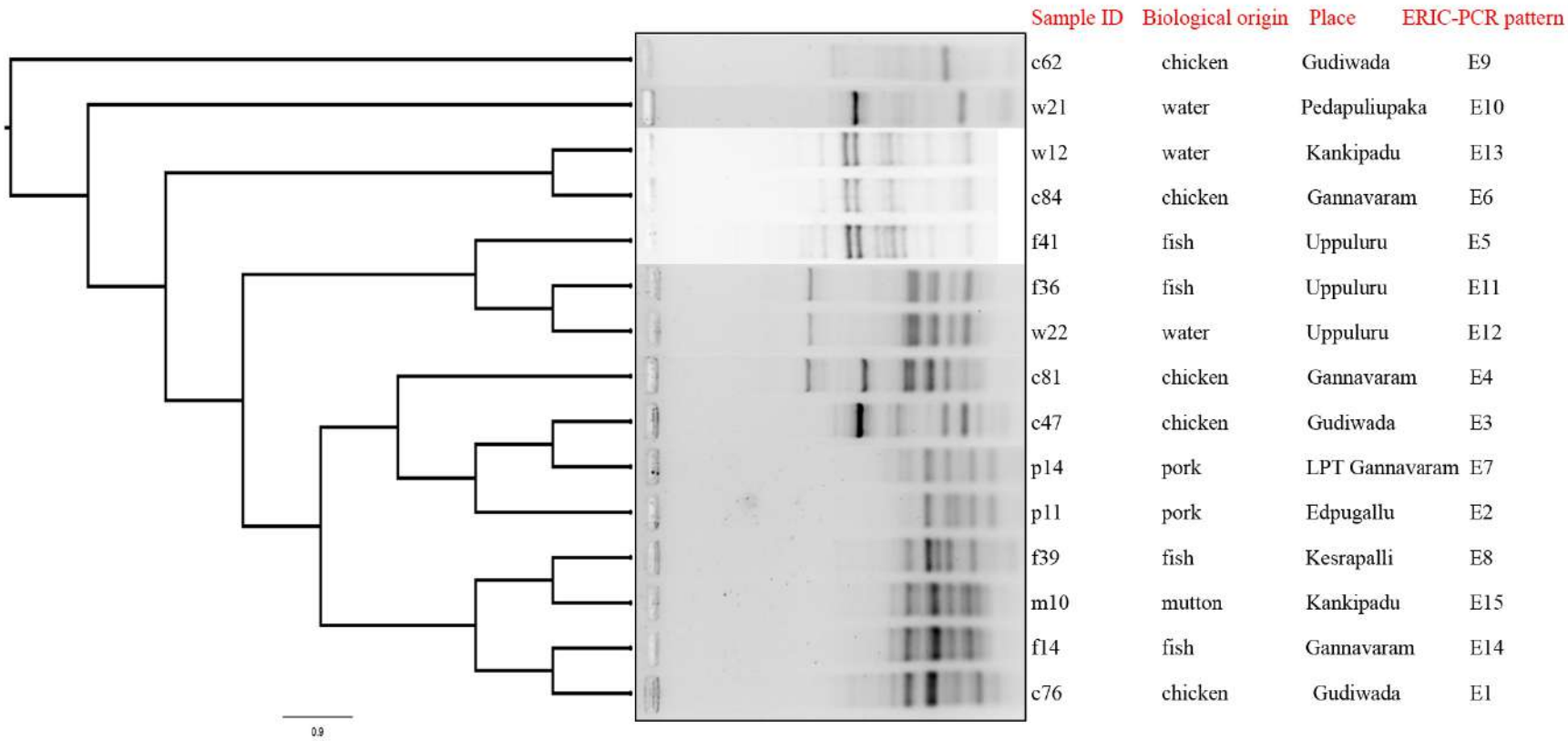


Fig-40 Dendrogram analysis of ERIC-PCR fingerprints of VR *E. faecium* isolates from different sources. The dendrogram was generated by the "Branch-and-bound" method using dollop program of PHYLIP 3.6 version

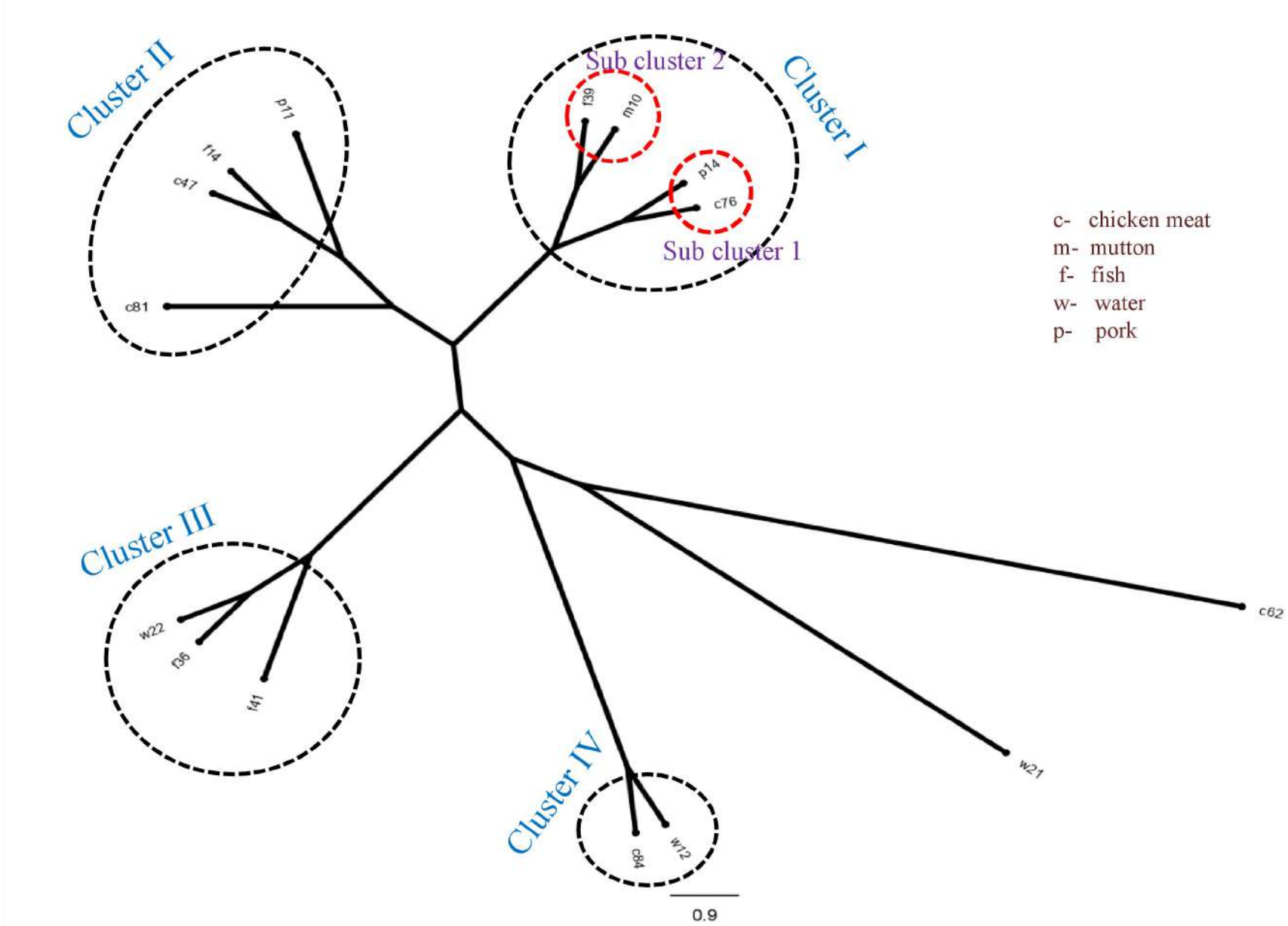


Fig-41: Cluster analysis of ERIC-PCR fingerprints of VR *E. faecium* isolates from different sources.
An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm)

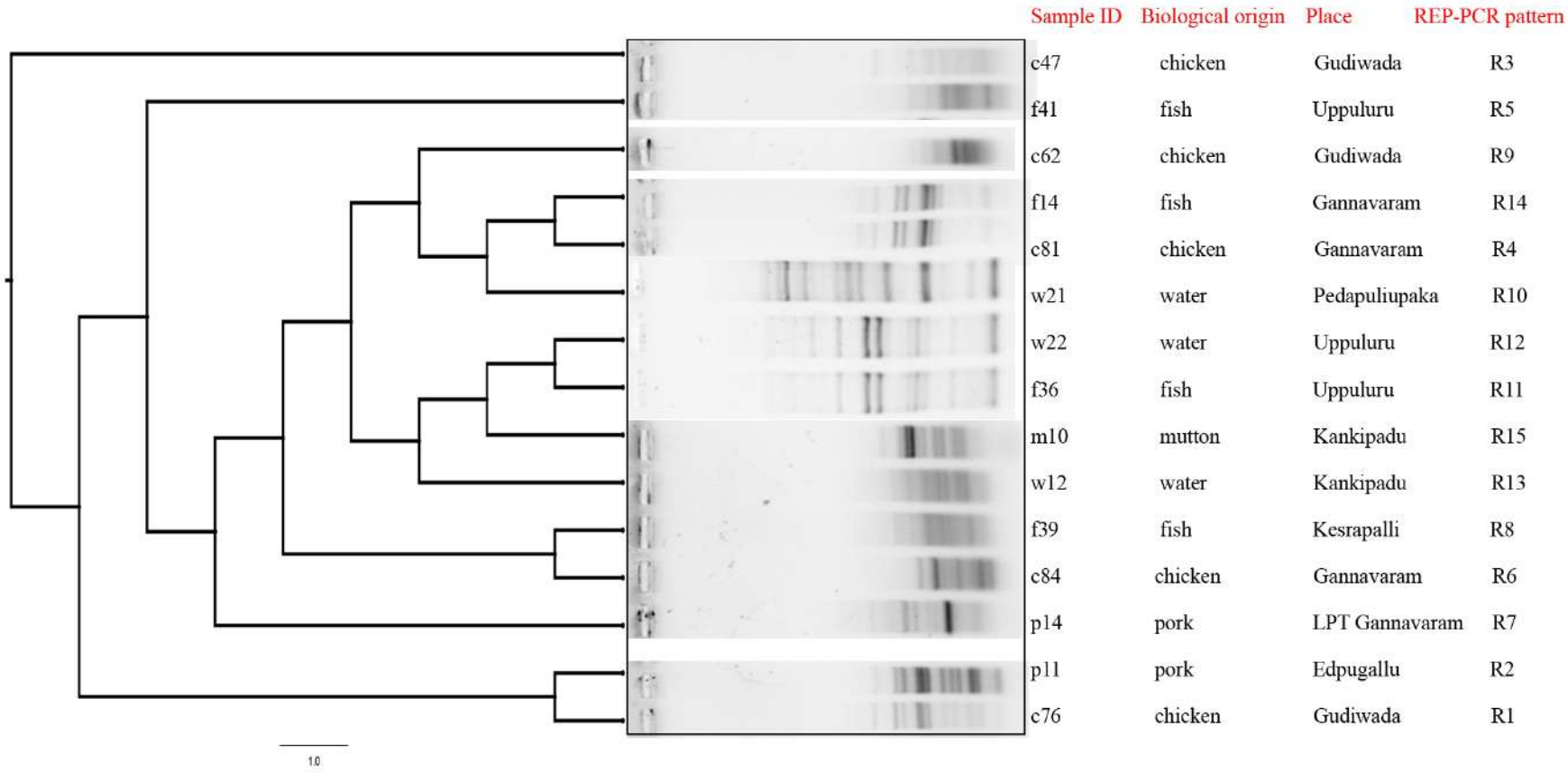


Fig-42 Dendrogram analysis of REP-PCR fingerprints of VR *E. faecium* isolates from different sources. The dendrogram was generated by the "Branch-and-bound" method using dollop program of PHYLIP 3.6 version

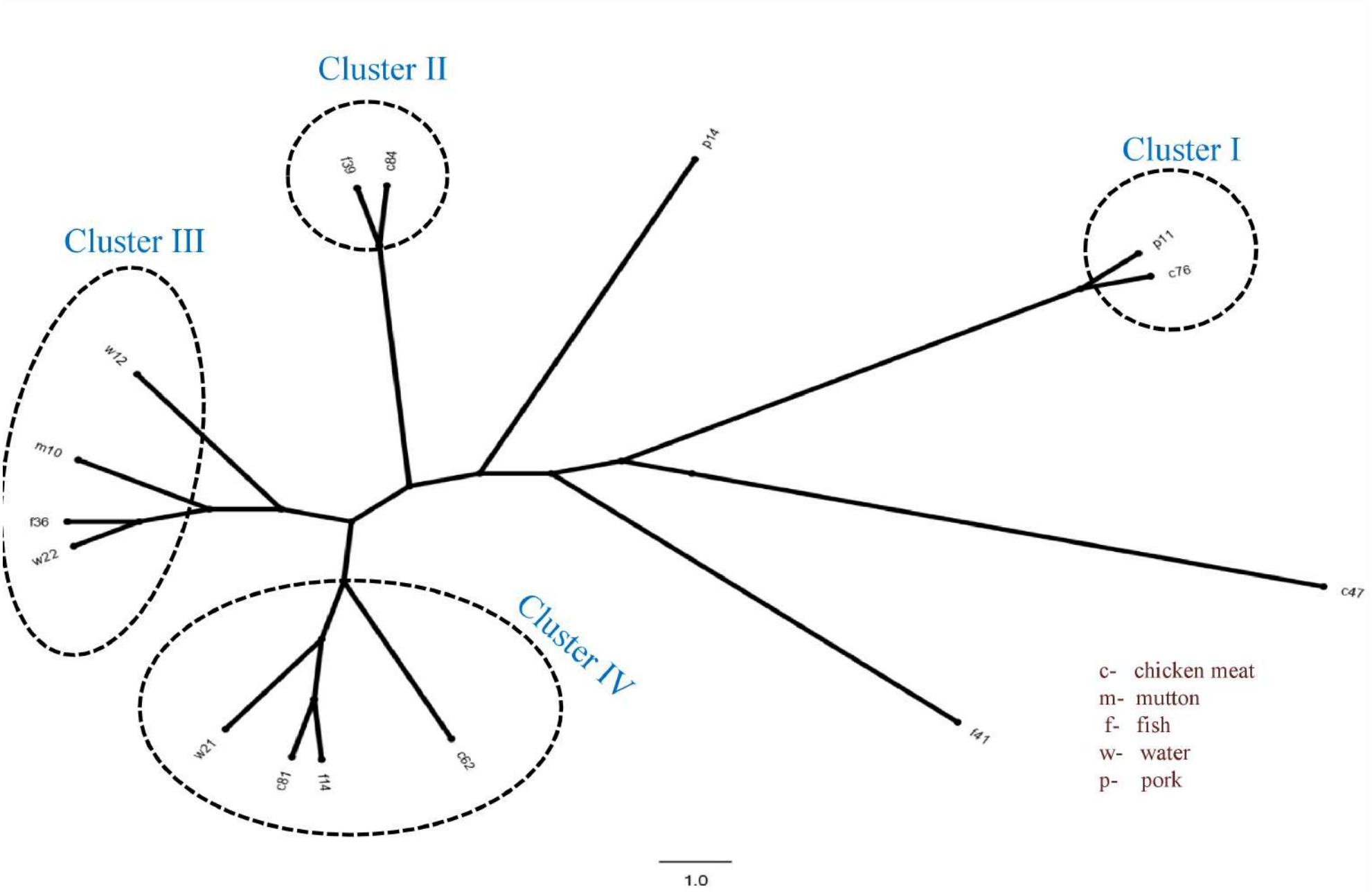


Fig-43: Cluster analysis of REP-PCR fingerprints of VR *E. faecium* isolates from different sources.
An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm)

any bands for both ERIC and REP-PCR. Of the 57 VR *E. gallinarum* analyzed, 57 ERIC-PCR patterns and 56 REP patterns were obtained. The binary score demonstrating the variety of 57 ERIC (E1-E57) and 56 REP PCR genotypes (R1-R56) were given in Table- 47 and 48, respectively. Two *E. gallinarum* isolates (c112 and c120) that had identical REP-PCR band pattern (R3) were distinguishable in ERIC-PCR pattern (E3 and E4). Dendrograms were constructed based on ERIC and REP-PCR profiles (Fig- 44 to 47) using dollop program of phylip 3.6 version.

Dendrogram analysis of ERIC-PCR profiles discriminated VR *E. gallinarum* isolates into six major clusters. Cluster I was divided into four sub clusters. Isolates c65, c31, m5 and h17 were sub clustered of which c31 and m5 showed 90% similarity. Within cluster I, quail isolate (q46) was distantly isolated from the four sub clusters. Sub clusters 2 and 3 have two isolates each (CC31 and p36; BR9 and q31, respectively) and sub cluster 4 has 3 isolates (c3, q27 and m52). Cluster II comprised of three sub clusters where sub cluster 1 has two isolates (cb17 and M79), sub cluster 2 have 3 isolates (c101, M19 and *E. gallinarum* MTCC7049) whereas M19 and *E. gallinarum* MTCC7049 were closely related with >90% similarity cut off and sub cluster 3 consisted of four isolates (cb11, CC10, c79 and M76). Cluster III has five isolates (M4, q50, c115, CC20 and hd5) and they were divided into two sub clusters where, human diarrhoeic isolate (hd5) and chicken cloacal isolate (CC20) expressed close genetic relatedness. Cluster IV comprised of three sub clusters where sub cluster 1 has M43, cb21 and q22, sub cluster 2 has six isolates (h20, M24, cb5, m26, hd1 and M77) where cb5 and m26 were closely related with >90% similarity cut off and sub cluster 3 has two isolates (c83 and PR6). Cluster V is the largest cluster consisting of 14 isolates and were grouped into five sub clusters where sub cluster 1 and 2 have two isolates each (c127 and CC13; c33 and m77, respectively), sub cluster 3 has c112, c99 and c153 isolates where c99 and c153 were closely related, sub cluster 4 has m25, c120 and w13

isolates of which c120 and m25 were closely related and sub cluster 5 has cb29, M3 and c159 isolates in which M3 and c159 were closely related. Within cluster V, chicken cloacal isolate (CC19) distantly away from other isolates that were sub clustered. Cluster VI consisted of two isolates, chicken (c88) and chicken cloacal origin (CC8) which showed great genetic relatedness. Five isolates (w18, m21, q36, M39 and q24) were found to be unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates from different sources.

Dendrogram analysis of REP-PCR profiles discriminated VR *E. gallinarum* isolates into a seven major clusters. Cluster I contained four isolates (c127, CC19, q22 and h17) where chicken cloacal isolate (CC17), showed closer genetic relation with isolate from quail (q22) origin. The clusters II and VII were the smallest clusters with three isolates each. Cluster II, human faecal isolate (h20) is distantly related with other two isolates (cb21 and M79). Cluster III comprised of three sub clusters where sub cluster 1 has five isolates (hd1, m25, c88, q36 and c3) and sub cluster 2 and 3 had two isolates each (w18 and M77; c101 and m77). Chicken cloacal isolate (CC13) was distantly related with other isolates of cluster III. Cluster IV was the largest cluster with 16 isolates and comprised of four sub clusters where sub cluster 1 (q46, M24, cb5 and m5), 2 (m26, c33, m52 and q27) and 4 (M43, c31, w13 and p36) had four isolates each and sub cluster 3 has three isolates (c112, c120 and cb29). Within the cluster IV, chicken cloacal origin isolate (CC8) showed distant relation with other isolates of the sub clusters. Cluster V was divided into four sub clusters where sub clusters 2 (c153, c65 and c159), 3 (m21, c79 and hd5) and 4 (CC19, c99 and M3) had three isolates each and sub cluster 1 has two isolates (q31 and BR9). Within the cluster V, *E. gallinarum* MTCC7049 showed distant relation with other isolates of the cluster. Cluster VI contained two sub clusters where sub cluster 1 has q24, M4 and M76 and sub cluster 2 has PR6, c115 and CC31 isolates. Cluster VII has three isolates (q50, cb17 and cb11)

Table 47: Scoring of VR E. *gallinarum* ERIC-PCR genotypes

Band pattern	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14	E15	E16
Sample id	h17	M79	c112	c120	c127	CC13	m26	hd1	w18	CC8	cb11	cb17	q24	h20	M19	M39
Band 1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
Band 2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Band 3	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Band 4	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
Band 5	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	0
Band 6	0	0	0	0	1	1	0	1	0	0	0	0	1	0	0	0
Band 7	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Band 8	0	1	1	1	0	0	1	0	0	0	0	0	0	1	1	0
Band 9	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
Band 10	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 11	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Band 12	1	0	1	1	1	1	0	0	1	1	0	0	1	0	1	1
Band 13	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
Band 14	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Band 15	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Band 16	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Band 17	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0
Band 18	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1
Band 19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 20	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Band 21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 23	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Band 24	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Band 25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 26	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
Band 27	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Band 28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 29	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Band 30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Band 31	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0
Total	**5**	**5**	**5**	**4**	**4**	**4**	**1**	**3**	**3**	**3**	**5**	**4**	**6**	**6**	**5**	**6**

Band pattern / Sample id	E17	E18	E19	E20	E21	E22	E23	E24	E25	E26	E27	E28	E29	E30	E31
	M4	M77	c115	c83	PR6	M43	q50	CC20	cb21	hd5	M3	m5	m52	m21	c3
Band 1	0	1	0	0	1	1	0	1	1	0	0	0	0	0	1
Band 2	0	0	0	0	1	0	0	0	0	0	0	1	1	0	1
Band 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 4	0	1	0	1	1	0	1	0	1	1	0	0	1	1	1
Band 5	1	0	0	1	0	0	1	0	0	1	0	0	1	1	0
Band 6	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1
Band 7	1	1	1	1	0	1	1	0	0	1	0	0	0	1	0
Band 8	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
Band 9	0	0	1	0	0	0	0	0	0	0	1	1	1	0	0
Band 10	0	1	0	1	1	0	0	0	0	0	1	0	0	1	1
Band 11	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1
Band 12	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Band 13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 14	1	1	0	0	1	1	0	0	0	0	0	0	1	0	1
Band 15	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Band 16	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1
Band 17	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Band 18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 19	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 20	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Band 21	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Band 22	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Band 23	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Band 24	0	1	0	1	1	0	0	0	0	1	0	0	0	1	1
Band 25	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
Band 26	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
Band 27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 28	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0
Band 29	1	0	0	1	1	0	0	0	0	0	0	0	0	1	1
Band 30	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 31	0	0	0	1	0	0	0	0	1	0	1	0	0	1	0
Total	**6**	**7**	**5**	**10**	**11**	**9**	**6**	**6**	**7**	**6**	**4**	**4**	**9**	**10**	**11**

(Contd…)

Band pattern / Sample id	E32 c79	E33 CC10	E34 CC19	E35 c31	E36 c33	E37 cb29	E38 w13	E39 CC31	E40 q46	E41 M76	E42 c159	E43 c65	E44 m77	E45 c101
Band 1	1	0	1	1	0	0	0	0	0	0	0		0	0
Band 2	0	0	0	0	0	0	1	0	1	1	0	1	1	1
Band 3	0	0	0	0	0	0	0	0	1	0	0	0	1	0
Band 4	0	1	0	1	1	0	0	0	1	0	0	0	0	1
Band 5	0	1	0	0	1	0	0	0	1	0	0	0	0	0
Band 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 7	1	1	0	0	1	0	0	0	0	0	0	0	0	0
Band 8	0	0	0	0	0	0	0	0	1	0	0	0	0	1
Band 9	0	0	0	0	0	1	1	0	0	1	1	1	1	0
Band 10	0	0	0	0	0	1	0	1	0	0	1	1	0	0
Band 11	1	1	1	1	1	0	1	0	1	1	0	0	0	1
Band 12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 13	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Band 14	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 15	0	0	0	0	0	0	0	1	1	0	0	0	0	1
Band 16	1	1	1	1	1	1	0	0	0	0	0	0	0	1
Band 17	1	0	0	0	0	0	0	0	0	1	0	0	0	0
Band 18	0	0	0	0	0	0	0	0	1	0	0	1	0	1
Band 19	0	0	1	0	0	0	0	1	0	0	1	0	0	0
Band 20	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Band 21	0	0	0	0	0	0	1	0	1	0	0	0	0	0
Band 22	1	0	0	0	0	0	0	0	0	0	0	1	0	0
Band 23	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 24	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Band 25	1	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 26	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 27	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 28	1	0	1	1	0	0	0	0	0	0	1	0	0	0
Band 29	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 30	0	0	1	0	0	0	0	1	0	0	0	0	1	0
Band 31	0	0	0	1	0	1	0	0	0	0	0	0	0	0
Total	**9**	**6**	**6**	**7**	**6**	**4**	**4**	**4**	**10**	**5**	**4**	**5**	**4**	**7**

(Contd…)

band pattern	E46	E47	E48	E49	E50	E51	E52	E53	E54	E55	E56	E57	MTCC
Sample id	p36	q27	cb5	M24	q36	c88	m25	q22	q31	BR9	c153	c99	MTCC
Band 1	0	0	0	0	0	0	0	0	1	0	0	0	0
Band 2	0	0	0	0	0	0	0	0	0	0	0	1	1
Band 3	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 4	0	0	0	0	0	1	0	1	1	1	1	0	0
Band 5	0	1	1	0	0	1	0	0	0	1	1	0	0
Band 6	0	0	0	1	1	1	0	1	0	0	0	0	0
Band 7	0	0	1	0	0	0	0	0	1	0	0	0	0
Band 8	0	1	0	1	1	0	0	1	0	1	1	1	1
Band 9	0	0	0	0	0	1	1	0	0	0	0	0	0
Band 10	1	0	0	0	0	0	0	0	0	1	1	1	0
Band 11	0	1	0	1	0	1	0	0	1	0	0	0	0
Band 12	0	0	0	0	1	1	1	0	0	0	0	1	1
Band 13	0	0	0	0	0	0	1	1	0	0	0	0	0
Band 14	1	1	1	0	0	0	0	0	0	1	1	0	0
Band 15	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 16	0	1	0	0	0	0	0	0	1	1	1	1	1
Band 17	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 18	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 19	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 20	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 21	0	0	0	0	0	0	0	0	1	1	0	0	0
Band 22	0	0	0	0	0	0	0	0	0	0	0	0	1
Band 23	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 24	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 25	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 26	1	1	0	0	1	0	0	0	0	0	0	0	0
Band 27	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 28	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 29	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 30	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 31	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	4	6	3	3	4	7	3	5	6	7	6	5	5

Isolates of chicken: E3, E4, E5, E19, E20, E31, E32, E35, E36, E42, E43, E45, E51, E56 and E57. **Isolates of mutton:** E7, E28, E29, E30, E44 and E52. **Isolates of milk:** E2, E15, E16, E17, E18, E22, E27, E41 and E49. **Isolates of quail:** E13, E23, E40, E47, E50, E53 and E54. **Isolates of pork:** E46. **Isolates of human faecal swabs:** E1 and E14. **Isolates of chicken cloacal swabs:** E6, E10, E24, E33, E34 and E39. **Isolates from water:** E9 and E38. **Isolates of human diarrhoea:** E8 and E26. **Isolates of carabeef:** E11, E12, E25, E37 and E48. **Isolates of pig rectal swab:** E21. **Isolates of buffalo rectal swab:** E55.

Number of VR *E. gallinarum* isolates : 57
Number of VR *E. gallinarum* ERIC-PCR genotypes: 57

Table-48 Scoring of *E. gallinarum* REP-PCR genotypes

Band pattern	R1	R2	R3	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15	R16	R17
Sample id	h17	M 79	c112	c120	c127	CC13	m26	hd1	w18	CC8	cb11	cb17	q24	h20	M19	M39	M4	M77
Band 1	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0
Band 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 3	1	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0
Band 4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Band 5	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0
Band 6	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 7	0	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0
Band 8	1	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1
Band 9	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 10	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Band 11	0	0	0	0	1	0	0	1	1	1	0	0	1	0	0	0	1	1
Band 12	1	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0	0	0
Band 13	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
Band 14	0	1	1	1	1	0	0	1	0	1	0	0	0	1	0	1	0	1
Band 15	1	0	0	0	0	1	1	0	0	0	0	0	1	1	1	0	1	0
Band 16	0	1	1	1	1	0	0	1	0	1	0	1	1	1	0	0	1	0
Band 17	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0
Band 18	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0
Band 19	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	1
Band 20	0	0	0	0	1	1	0	0	0	0	0	0	1	1	1	0	0	0
Band 21	0	1	0	0	0	0	1	1	0	1	1	1	0	0	0	0	0	0
Band 22	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0
Band 23	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
Band 24	1	1	1	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0
Band 25	0	0	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0	1
Band 26	0	0	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0
Band 27	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0
Band 28	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Band 29	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0
Band 30	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	1	0
Band 31	0	1	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	0
Band 32	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Band 33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 35	0	0	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0
Band 36	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0
Band 37	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 39	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0
Band 40	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1
Band 41	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0
Band 42	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
Band 43	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 45	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0
Band 46	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Band 47	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Band 48	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Band 49	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0
Band 50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 51	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Band 52	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 53	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 54	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 55	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
TOTAL	**10**	**9**	**10**	**10**	**13**	**11**	**8**	**11**	**10**	**12**	**10**	**10**	**10**	**14**	**9**	**5**	**7**	**8**

(contd..)

Band pattern	R18	R19	R20	R21	R22	R23	R24	R25	R26	R27	R28	R29	R30	R31	R32	R33
Sample id	c115	c83	PR6	M43	q50	CC20	cb21	hd5	M3	m5	m52	m21	c3	c79	CC10	CC19
Band 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 3	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
Band 4	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0
Band 5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Band 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Band 7	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 8	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1
Band 9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 10	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	0
Band 11	1	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1
Band 12	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1
Band 13	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0
Band 14	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 15	0	0	0	0	0	0	0	1	1	0	1	1	1	0	1	0
Band 16	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Band 17	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0
Band 18	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Band 19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 20	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0
Band 21	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0
Band 22	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0
Band 23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 25	0	0	0	0	1	0	0	1	1	0	0	1	0	0	1	0
Band 26	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
Band 27	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0
Band 28	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Band 29	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Band 30	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0
Band 31	0	0	0	0	0	1	1	1	1	0	0	0	0	1	0	0
Band 32	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0
Band 33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 34	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
Band 35	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Band 36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 37	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 39	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 40	1	1	1	0	0	0	0	0	0	0	0	1	0	1	0	0
Band 41	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0
Band 42	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 43	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 45	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 46	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
Band 47	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 49	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 50	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 51	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Band 52	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 53	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Band 54	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 55	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0
Band 56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TOTAL	**7**	**9**	**9**	**4**	**7**	**5**	**6**	**4**	**11**	**3**	**4**	**10**	**5**	**9**	**9**	**4**

(contd..)

band pattern / Sample id	R34 c31	R35 c33	R36 cb29	R37 w13	R38 CC31	R39 q46	R40 M76	R41 c159	R42 c65	R43 m77	R44 c101	R45 p36	R46 q27	R47 cb5
Band 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 2	0	0	0	0	0	1	0	1	0	0	0	0	0	0
Band 3	0	0	0	0	0	0	0	0	0	1	0	0	0	1
Band 4	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 6	0	0	0	0	0	0	0	1	0	0	0	1	1	0
Band 7	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 8	0	0	0	1	1	1	1	0	0	0	0	0	0	0
Band 9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 10	0	1	0	0	0	0	0	0	1	1	1	0	0	0
Band 11	0	0	1	0	0	1	1	1	0	0	0	0	1	1
Band 12	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 13	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 14	0	0	0	1	0	1	1	1	0	0	0	0	0	0
Band 15	0	1	1	0	0	0	0	1	1	1	1	0	0	0
Band 16	0	0	1	0	0	0	0	1	0	0	0	0	1	1
Band 17	0	0	0	1	0	1	1	0	1	1	1	0	1	1
Band 18	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 19	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 20	0	0	1	0	0	1	1	0	0	0	0	0	0	0
Band 21	1	1	0	1	0	0	0	0	1	1	1	0	0	0
Band 22	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 23	0	0	0	0	0	0	0	0	1	1	1	0	0	1
Band 24	1	0	1	0	0	0	0	0	0	0	0	0	0	0
Band 25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 26	0	0	0	0	0	1	1	1	0	0	0	0	0	0
Band 27	0	0	0	1	1	0	0	0	0	0	0	0	0	0
Band 28	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Band 29	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 30	0	0	1	0	0	0	0	1	0	0	0	0	0	0
Band 31	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 32	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 33	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 34	0	0	0	0	0	1	1	0	0	0	0	0	0	0
Band 35	0	1	0	0	0	0	0	1	0	0	0	0	0	0
Band 36	1	0	0	0	0	0	0	0	0	1	1	0	0	0
Band 37	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 38	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 39	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 40	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 41	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 42	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 43	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 44	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 45	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 46	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 47	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 48	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 49	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 50	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 51	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 52	0	1	0	0	0	0	1	0	0	0	0	0	0	0
Band 53	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Band 54	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 55	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 56	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TOTAL	**8**	**9**	**6**	**5**	**3**	**8**	**10**	**9**	**5**	**7**	**6**	**1**	**4**	**5**

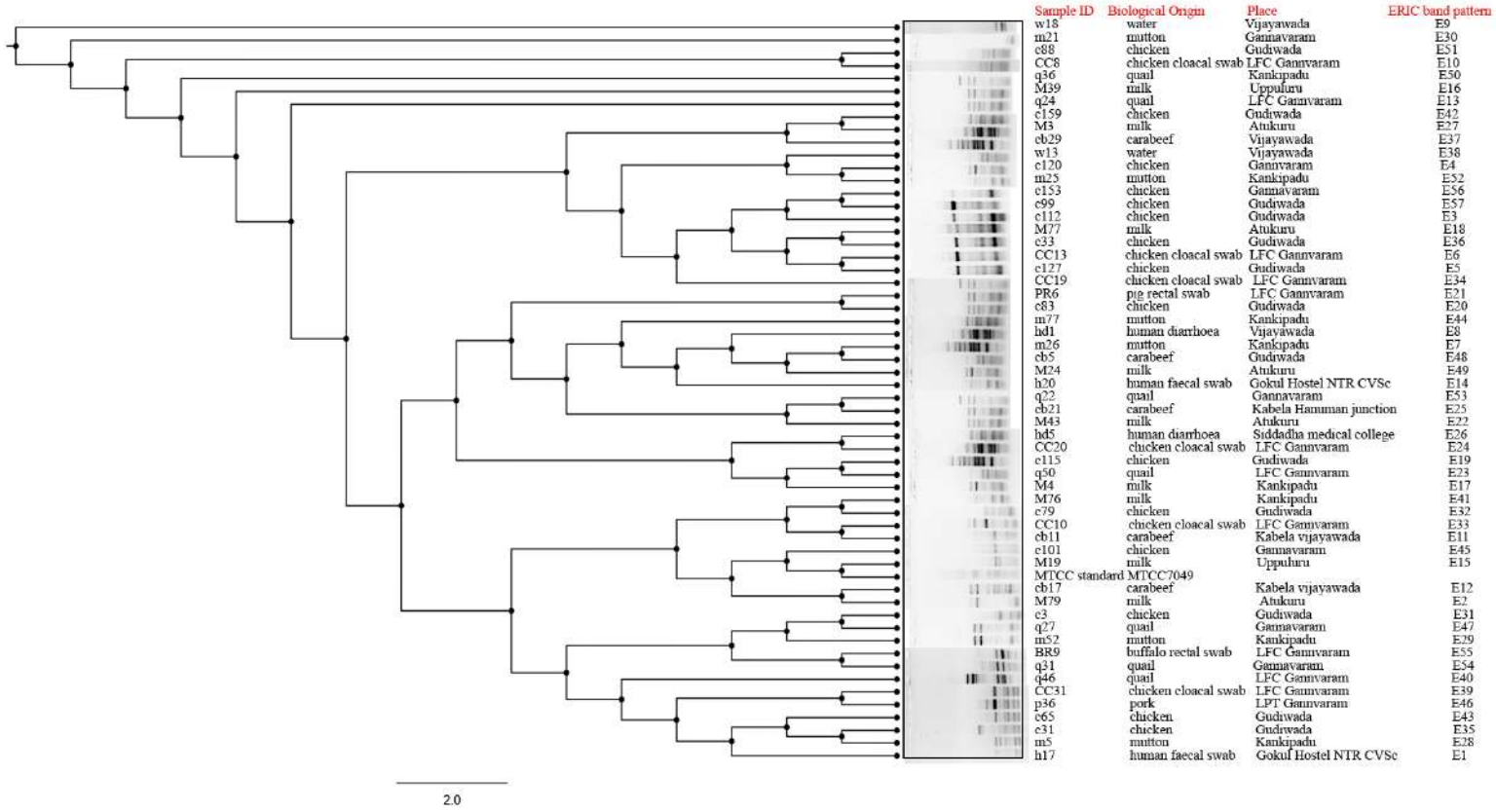
(contd..)

Band pattern	R48	R49	R50	R51	R52	R53	R54	R55	R56	MTCC
Sample id	M24	q36	c88	m25	q22	q31	BR9	c153	c99	MTCC
Band 1	0	0	0	0	0	0	0	0	0	0
Band 2	0	1	0	0	0	1	0	0	0	0
Band 3	0	1	0	0	1	1	0	0	1	1
Band 4	0	0	1	0	0	0	1	0	1	1
Band 5	0	0	0	0	0	0	0	0	0	0
Band 6	0	0	0	1	0	0	0	1	1	1
Band 7	0	0	1	0	1	0	1	0	0	0
Band 8	1	0	0	0	0	0	0	0	0	1
Band 9	0	0	0	0	0	0	0	0	0	0
Band 10	0	0	1	1	0	0	1	1	0	0
Band 11	0	0	0	0	1	0	0	0	1	1
Band 12	0	0	0	0	0	0	0	0	0	0
Band 13	0	0	0	0	0	0	0	0	0	0
Band 14	1	0	1	1	1	0	1	1	1	1
Band 15	1	0	0	1	0	0	0	1	1	1
Band 16	0	0	0	0	0	0	0	0	0	0
Band 17	0	0	0	0	0	0	0	0	0	0
Band 18	0	0	0	0	0	0	0	0	0	0
Band 19	0	0	0	0	0	0	0	0	0	0
Band 20	0	0	0	0	0	0	0	0	0	0
Band 21	0	0	0	0	0	0	0	0	0	0
Band 22	0	0	0	0	0	0	0	0	0	0
Band 23	1	0	0	0	0	0	0	0	0	0
Band 24	0	0	0	1	0	0	0	1	0	0
Band 25	0	0	0	0	0	0	0	0	0	0
Band 26	1	0	0	1	1	0	0	1	1	1
Band 27	0	0	0	0	0	0	0	0	0	0
Band 28	0	0	0	0	0	0	0	0	0	0
Band 29	0	0	0	0	0	0	0	0	1	1
Band 30	0	0	0	1	1	0	0	1	0	0
Band 31	0	0	0	0	0	0	0	0	0	0
Band 32	0	0	0	0	0	0	0	0	0	0
Band 33	0	0	0	0	0	0	0	0	0	0
Band 34	0	0	0	0	0	0	0	0	0	0
Band 35	0	0	0	0	0	0	0	0	0	0
Band 36	0	0	0	0	0	0	0	0	0	0
Band 37	0	0	0	0	0	0	0	0	0	0
Band 38	0	0	0	0	0	0	0	0	0	0
Band 39	0	0	0	0	0	0	0	0	0	0
Band 40	0	0	0	0	0	0	0	0	0	0
Band 41	0	0	0	0	0	0	0	0	0	0
Band 42	0	0	0	0	0	0	0	0	0	0
Band 43	0	0	0	0	0	0	0	0	0	0
Band 44	0	0	0	0	0	0	0	0	0	0
Band 45	0	0	0	0	0	0	0	0	0	0
Band 46	0	0	0	0	0	0	0	0	0	0
Band 47	0	0	0	0	0	0	0	0	0	0
Band 48	0	0	0	0	1	0	0	0	0	0
Band 49	0	0	0	0	0	0	0	0	0	0
Band 50	1	0	0	0	0	0	0	0	0	0
Band 51	0	0	0	0	0	0	0	0	0	0
Band 52	0	0	0	0	0	0	0	0	0	0
Band 53	0	0	0	0	0	0	0	0	0	0
Band 54	0	0	0	0	0	0	0	0	0	0
Band 55	0	0	0	0	0	0	0	0	0	0
Band 56	0	0	0	0	0	0	0	0	0	0
TOTAL	6	2	4	7	7	2	4	7	8	9

Isolates of chicken: R3, R3, R4, R18, R19, R30, R31, R34, R36, R41, R42, R44, R50, R55 and R56. **Isolates of mutton:** R6, R27, R28, R29, R43 and R51. **Isolates of milk:** R2, R14, R15, R16, R17, R21, R26, R40 and R48. **Isolates of quail:** R12, R22, R39, R46, R49, R52 and R53. **Isolates of pork: R**45. **Isolates of human faecal swabs:** R1 and R13. **Isolates of chicken cloacal swabs:** R5, R9, R23, R32, R33 and R38. **Isolates from water:** R8 and R37. **Isolates of human diarrhoea:** R7 and R25. **Isolates of carabeef:** R10, R11, R24, R36 and R47. **Isolates of pig rectal swab:** R20. **Isolates of buffalo rectal swab:** R54.

Indistinguishable REP-PCR pattern R3 which had distinguishable ERIC-PCR patterns E3 and E4.

Number of VR *E. gallinarum* isolates : 57
Number of VR *E. gallinarum* REP-PCR genotypes: 56



Sample ID	Biological Origin	Place	ERIC band pattern
w18	water	Vijayawada	E9
m21	mutton	Gannavaram	E30
c88	chicken	Gudiwada	E51
CC8	chicken cloacal swab	LFC Gannvaram	E10
q36	quail	Kankipadu	E50
M39	milk	Uppuluru	E16
q24	quail	LFC Gannvaram	E13
c159	chicken	Gudiwada	E42
M3	milk	Atukuru	E27
cb29	carabeef	Vijayawada	E37
w13	water	Vijayawada	E38
c120	chicken	Gannvaram	E4
m25	mutton	Kankipadu	E52
c153	chicken	Gannavaram	E56
c99	chicken	Gudiwada	E57
c112	chicken	Gudiwada	E3
M77	milk	Atukuru	E18
c33	chicken	Gudiwada	E36
CC13	chicken cloacal swab	LFC Gannvaram	E6
c127	chicken	Gudiwada	E5
CC19	chicken cloacal swab	LFC Gannvaram	E34
PR6	pig rectal swab	LFC Gannvaram	E21
c83	chicken	Gudiwada	E20
m77	mutton	Kankipadu	E44
hd1	human diarrhoea	Vijayawada	E8
m26	mutton	Kankipadu	E7
cb5	carabeef	Gudiwada	E48
M24	milk	Atukuru	E49
h20	human faecal swab	Gokul Hostel NTR CVSc	E14
q22	quail	Gannavaram	E53
cb21	carabeef	Kabela Hanuman junction	E25
M43	milk	Atukuru	E22
hd5	human diarrhoea	Siddadha medical college	E26
CC20	chicken cloacal swab	LFC Gannvaram	E24
c115	chicken	Gudiwada	E19
q50	quail	LFC Gannvaram	E23
M4	milk	Kankipadu	E17
M76	milk	Kankipadu	E41
c79	chicken	Gudiwada	E32
CC10	chicken cloacal swab	LFC Gannvaram	E33
cb11	carabeef	Kabela vijayawada	E11
c101	chicken	Gannavaram	E45
M19	milk	Uppuluru	E15
MTCC standard	MTCC7049		
cb17	carabeef	Kabela vijayawada	E12
M79	milk	Atukuru	E2
c3	chicken	Gudiwada	E31
q27	quail	Gannavaram	E47
m52	mutton	Kankipadu	E29
BR9	buffalo rectal swab	LFC Gannvaram	E55
q31	quail	Gannavaram	E54
q46	quail	LFC Gannvaram	E40
CC31	chicken cloacal swab	LFC Gannvaram	E39
p36	pork	LPT Gannvaram	E46
c65	chicken	Gudiwada	E43
c31	chicken	Gudiwada	E35
m5	mutton	Kankipadu	E28
h17	human faecal swab	Gokul Hostel NTR CVSc	E1

Fig-44 Dendrogram analysis of ERIC-PCR fingerprints of VR *E. gallinarum* isolates from different sources. The dendrogram was generated by the "Branch-and-bound" method using dollop program of PHYLIP 3.6 version

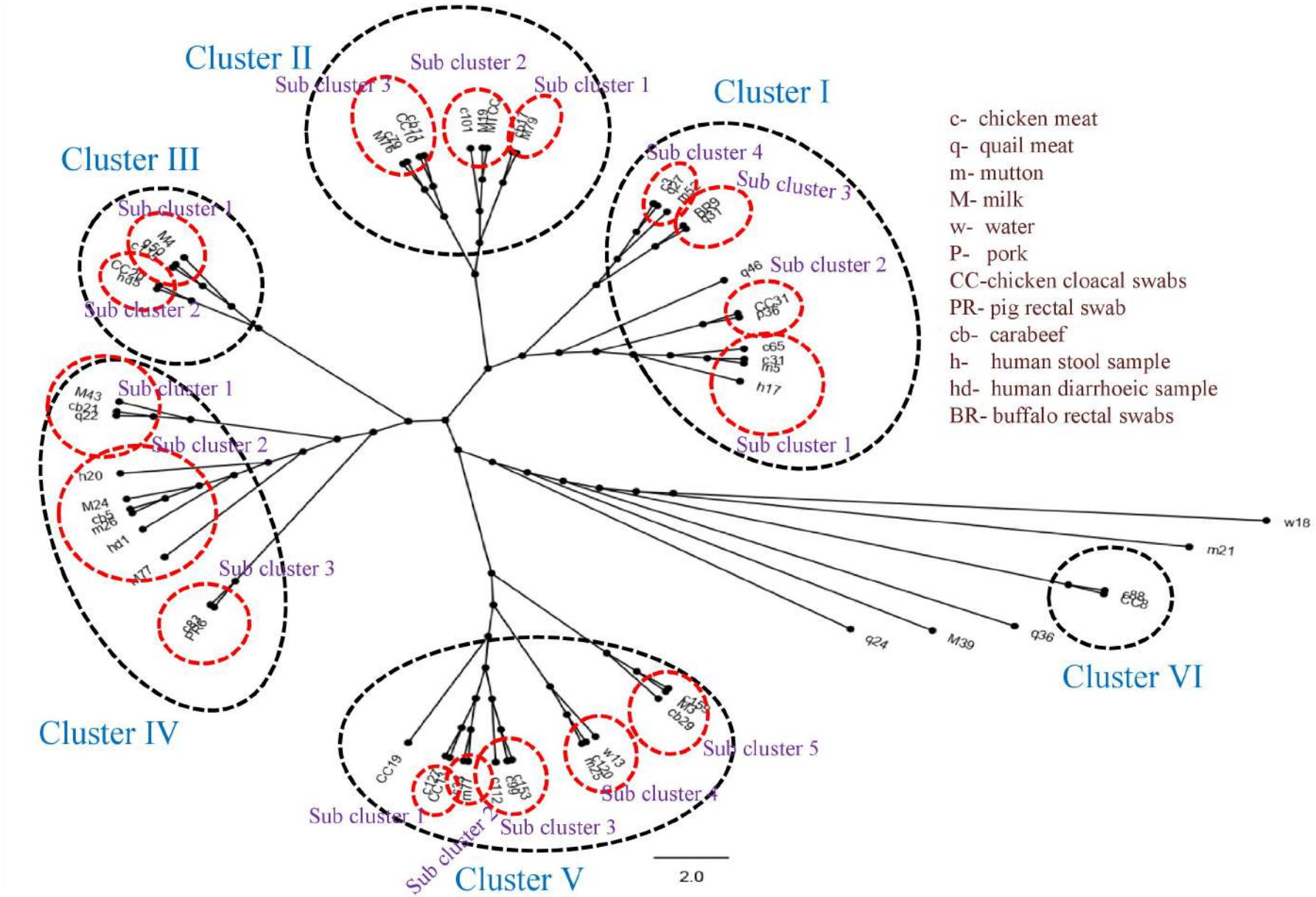


Fig-45: Cluster analysis of ERIC-PCR fingerprints of VR *E. gallinarum* isolates from different sources.
An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm)

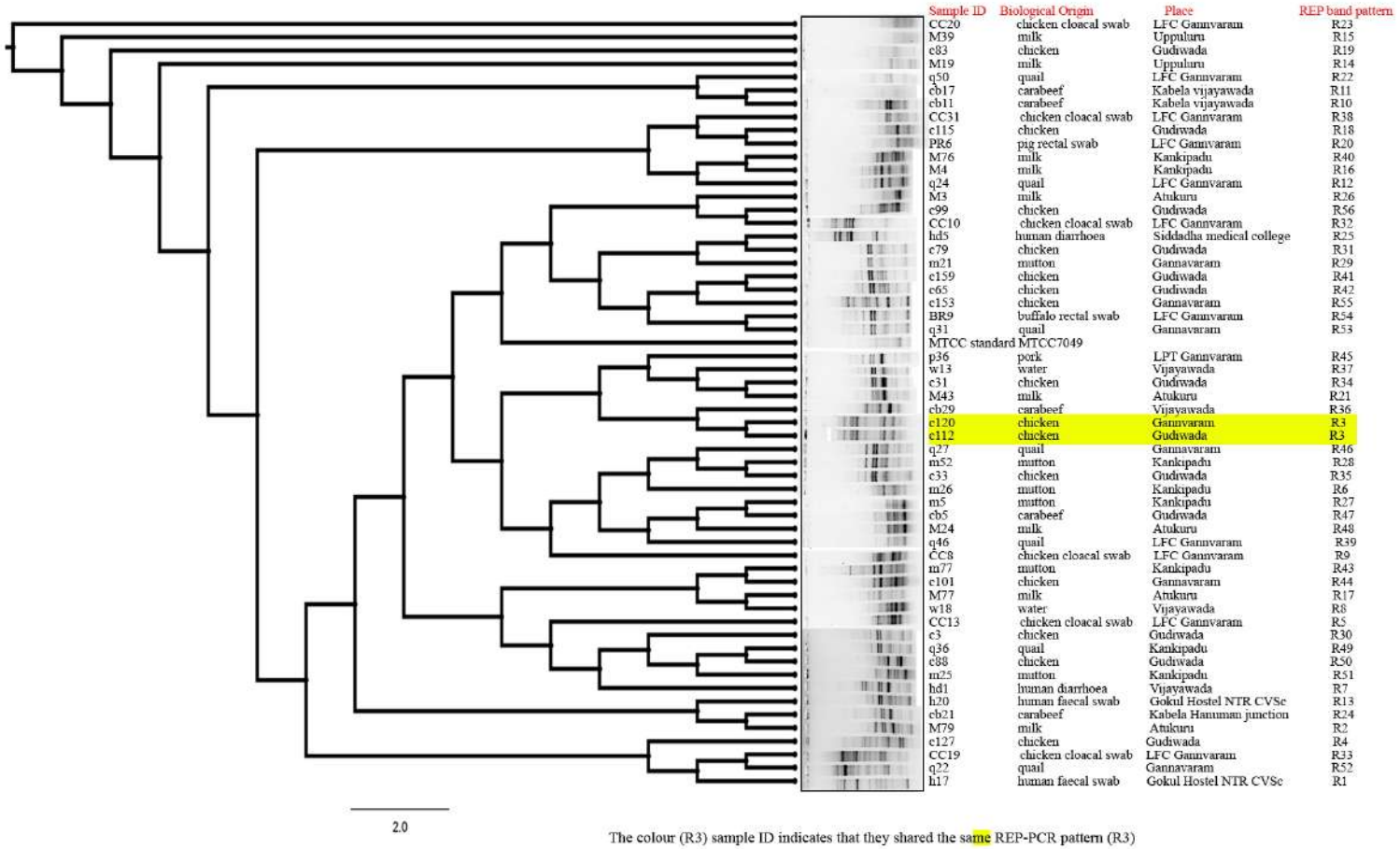


Fig-46 Dendrogram analysis of REP-PCR fingerprints of VR *E. gallinarum* isolates from different sources.
The dendrogram was generated by the "Branch-and-bound" method using dollop program of PHYLIP 3.6 version

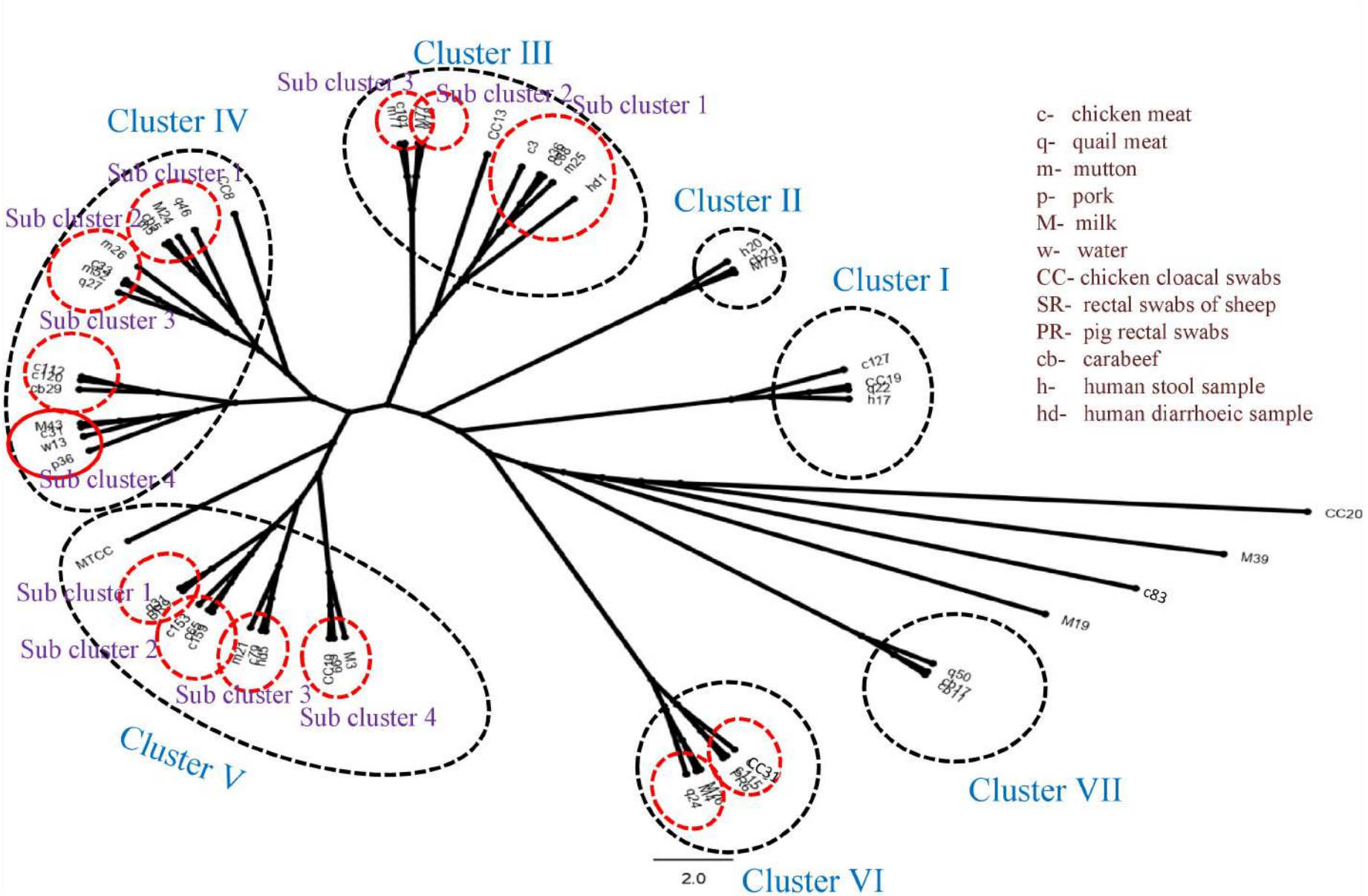


Fig-47: Cluster analysis of REP-PCR fingerprints of VR *E. gallinarum* isolates from different sources.
An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm)

where quail isolate (q50) clustered separately from isolates of carabeef origin (cb17 and cb11). Four isolates (CC20, M39, c83 and M19) were found to be unclustered (UC) with other isolates.

4.8.4 Genetic diversity of VR *E. casseliflavus* isolates

ERIC-PCR typing revealed 2-12 fragments per isolate, ranging in size from ~100 bp to ~2000 bp, whereas REP-PCR typing revealed 3-12 fragments resolved per isolate, ranging in size from ~100 bp to ~2000 bp. Of the 31 VR *E. casseliflavus* analyzed, 31 ERIC-PCR patterns and 31 REP patterns were obtained. The binary score demonstrating the variety of 31 ERIC (E1-E31) and 31 REP PCR genotypes (R1-R31) were given in Table 49 and 50, respectively. Dendrograms were constructed based on ERIC and REP-PCR profiles (Fig-48 to 51) using dollop program of phylip 3.6 version.

Dendrogram analysis of ERIC-PCR profiles discriminated VR *E. casseliflavus* isolates into four major clusters. Cluster I comprised of two sub clusters where sub cluster 1 has c142, SR8 and M8 isolates and sub cluster 2 has three isolates of quail origin (q2, q50 and q15) showing close genetic relatedness. Cluster II comprised of two sub clusters where sub cluster 1 has c27, c94 and c66 (isolates of chicken origin) and sub cluster 2 has CC17, m6 and c109. Within cluster II, isolate of chicken origin (c139), genetically far distant from other isolates of the cluster. Cluster III was divided into three sub clusters where sub cluster 1 has q5, q49, m30 and c122, where isolate of chicken origin (c122) arranged distantly from other three isolates, sub cluster 2 has w10 and f13 and sub cluster 3 has c56, SR6 and c32. Cluster IV again divided into two sub clusters and each with two isolates (c11 and c13; f35 and m39). Five isolates (c119, q44, c93, c25 and c146) were found to be unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates.

Dendrogram analysis of REP-PCR profiles discriminated VR *E. casseliflavus* isolates into seven major clusters. Clusters I was grouped into two sub clusters where sub cluster 1 has c142, w10 and SR8 and sub cluster 2 has m39 and c25. Cluster II again divided into two sub clusters having isolates of chicken (c11 and c32) and isolates of chicken and quail origin (c122, c146 and q15). Cluster III comprised of two sub clusters where sub cluster 1 has q49 and c94 and sub cluster 2 has m30 and c27. Similarly cluster IV again divided into two sub clusters where sub cluster 1 has c13 and f35 and sub cluster 2 has m8 and c139. Cluster V contained isolates of chicken (c93 and c66) and quail origin (q50) where c66 and q50 isolates showed great genetic relatedness. Cluster VI had four isolates (q44, c119, CC17 and c109) of which fc17 and c119 were showing close genetic association. Cluster VII has three isolates (SR6, f13 and c56) where isolates SR6 and f13 were showing close genetic relation (>90% similarity cut off value). Three isolates (q5, M6 and q2) were found to be unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates.

Table-49 Scoring of VR *E. casseliflavus* ERIC-PCR genotypes

Band pattern	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14	E15	E16	E17	E18	E19	E20	E21	E22	E23	E24	E25	E26	E27	E28	E29	E30	E31
Sample id	c142	c25	m6	c11	c13	f35	m8	w10	SR8	q2	q15	q44	c119	c109	c94	c32	c66	q49	m30	c27	c93	c122	c56	f13	SR6	CC17	c139	m39	q50	c146	q5
Band 1	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
Band 2	0	0	1	1	1	0	0	0	0	0	0	1	0	1	1	0	1	0	0	1	1	0	0	0	1	0	0	0	0	1	0
Band 3	1	0	0	0	0	1	1	1	0	1	1	0	1	0	0	1	0	0	0	0	0	1	1	1	1	0	1	1	0	0	1
Band 4	1	0	1	0	1	1	0	1	0	1	1	1	0	1	1	0	0	0	0	1	0	0	1	1	0	1	0	1	1	0	1
Band 5	1	0	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
Band 6	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	1
Band 7	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	0	0	0	1	0	0
Band 8	1	1	1	1	1	0	1	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 9	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 10	0	0	0	0	0	0	1	1	0	1	1	0	1	1	1	0	1	1	1	1	0	0	0	1	0	0	1	1	1	0	1
Band 11	0	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 12	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0
Band 13	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	1	1	1	0	0	0	0
Band 14	1	0	0	0	0	0	1	0	0	1	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
Band 15	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1	1	0	1
Band 16	0	0	1	1	1	1	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Band 17	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	1	0	0	0	0	0	0
Band 18	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Band 19	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1
Band 20	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 21	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Band 22	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Band 24	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	1
Band 25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
Band 26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Band 27	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 29	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
Band 31	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 32	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	1	1	0	0	0
Band 33	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 34	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 35	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TOTAL	7	6	7	8	12	8	8	6	6	7	8	8	7	5	6	2	3	5	5	9	4	8	7	5	6	7	8	8	8	3	7

Isolates of chicken: E1, E2, E4, E5, E13, E14, E15, E16, E17, E20, E21, E22, E23, E27 and E30. **Isolates of mutton:** E3, E19 and E28. **Isolates of fish:** E6 and E24. **Isolates of quail:** E10, E11, E12, E18, E29 and E31. **Isolates of milk:** E7. **Isolates of sheep rectal swabs:** E9 and E25. **Isolates of chicken cloacal swabs:** E26. **Isolates from water:** E8.

Number of VR *E. casseliflavus* isolates : 31
Number of VR *E. casseliflavus* ERIC-PCR genotypes: 31

Table-50 Scoring of *E. casseliflavus* REP-PCR genotypes

Band pattern	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15	R16
Sample id	c142	c25	m6	c11	c13	f35	m8	w10	SR8	q2	q15	q44	c119	c109	c94	c32
Band 1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
Band 2	1	0	1	1	1	1	0	0	1	0	0	0	0	0	0	0
Band 3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Band 4	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
Band 5	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 6	0	1	0	0	1	1	1	0	1	0	0	1	0	1	0	0
Band 7	1	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0
Band 8	0	0	0	0	1	1	1	0	0	0	0	1	0	1	1	0
Band 9	1	0	0	1	0	0	1	1	0	0	1	0	0	0	0	1
Band 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 11	1	0	0	0	0	0	0	1	0	0	1	1	1	1	0	1
Band 12	0	0	0	1	1	1	0	0	0	1	0	0	0	0	1	0
Band 13	1	0	1	0	1	1	1	0	1	0	1	1	1	0	1	0
Band 14	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Band 15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 16	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Band 17	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
Band 18	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
Band 19	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0
Band 20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Band 21	0	1	1	0	0	0	0	0	0	1	0	0	1	1	0	1
Band 22	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0
Band 23	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0
Band 24	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 25	0	0	0	1	0	0	0	1	1	0	1	0	0	0	1	0
Band 26	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0
Band 27	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1
Band 28	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0
Band 29	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 30	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
Band 31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 32	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
Band 33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 34	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Band 36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 37	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Band 39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 40	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1
Band 41	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 42	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 43	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 44	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
TOTAL	6	8	7	6	9	6	7	7	7	6	7	10	6	7	7	8

Band pattern	R17	R18	R19	R20	R21	R22	R23	R24	R25	R26	R27	R28	R29	R30	R31
Sample id	c66	q49	m30	c27	c93	c122	c56	f13	SR6	CC17	c139	m39	q50	c146	q5
Band 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Band 3	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0
Band 4	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Band 5	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
Band 6	0	0	0	1	0	0	1	1	1	1	1	0	0	0	0
Band 7	0	1	0	0	0	0	1	1	1	0	0	0	0	0	0
Band 8	1	1	0	1	1	0	0	0	0	1	1	0	1	0	1
Band 9	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Band 10	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
Band 11	1	0	0	0	1	0	0	0	0	0	1	1	1	0	0
Band 12	0	1	1	1	0	0	1	1	1	0	0	0	1	1	0
Band 13	0	1	1	1	1	1	0	0	0	0	1	0	0	0	0
Band 14	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Band 15	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0
Band 16	0	1	1	0	0	1	0	1	0	0	0	0	1	0	0
Band 17	1	0	0	0	0	0	1	0	1	1	0	0	0	0	1
Band 18	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Band 19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 20	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
Band 21	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Band 22	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0
Band 23	1	1	0	0	0	0	0	0	0	0	1	1	1	1	0
Band 24	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0
Band 25	1	1	0	1	1	1	0	0	0	1	1	1	1	0	1
Band 26	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Band 27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 28	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
Band 29	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
Band 30	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0
Band 31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Band 32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 33	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Band 34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Band 37	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Band 38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 39	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 40	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
Band 41	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
Band 42	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Band 43	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Band 44	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
TOTAL	8	12	8	11	8	10	8	6	6	5	8	8	8	3	7

Isolates of chicken: R1, R2, R4, R5, R13, R14, R15, R16, R17, R20, R21, R22, R23, R27 and R30. **Isolates of mutton:** R7, R19 and R28. **Isolates of fish:** R6 and R24. **Isolates of quail:** R10, R11, R12, R18, R29 and R31. **Isolates of milk:** R3. **Isolates of sheep rectal swabs:** R9 and R25. **Isolates of chicken cloacal swabs:** R26. **Isolates from water:** R8.

Number of VR *E. casseliflavus* isolates : 31
Number of VR *E. casseliflavus* REP-PCR genotypes: 31

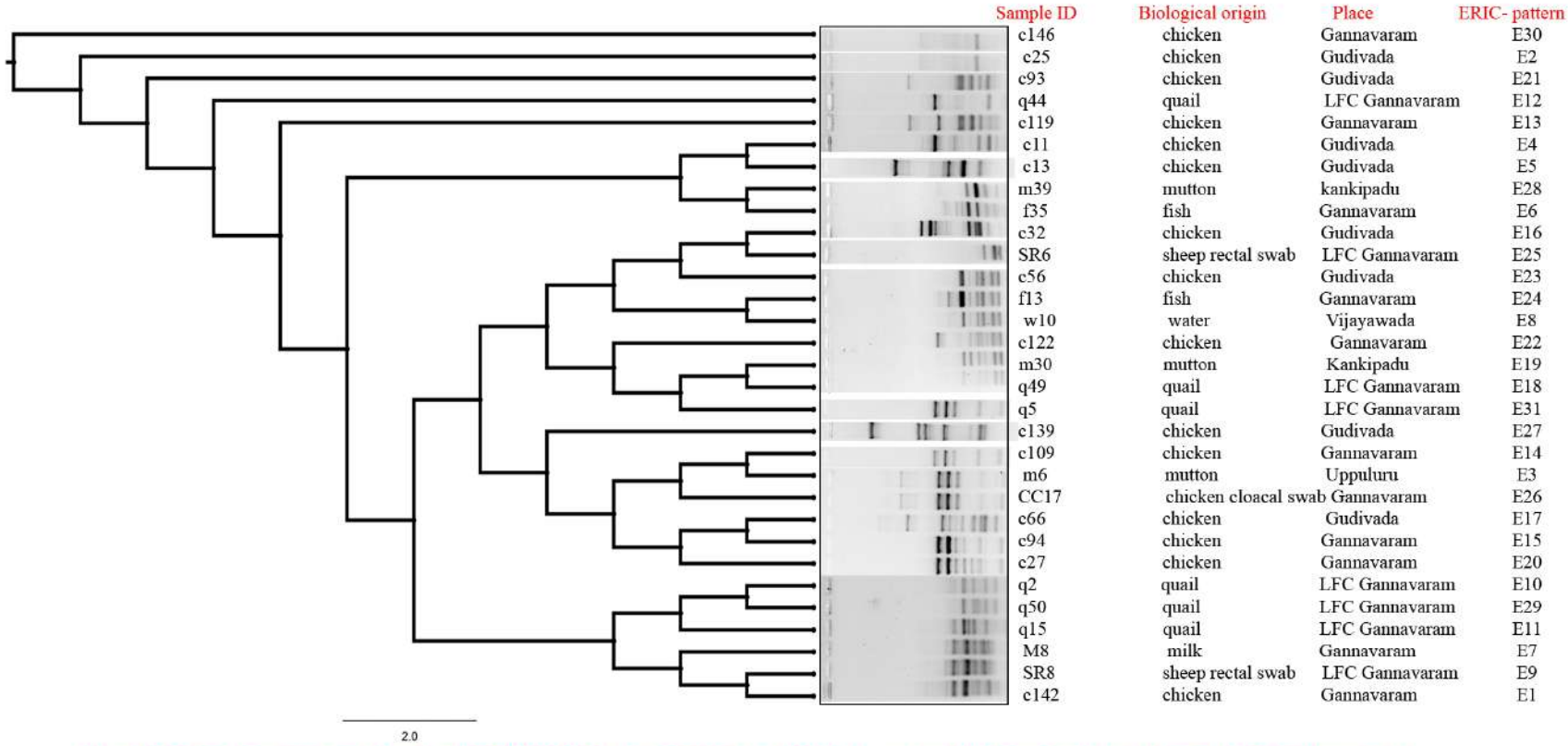


Fig-48 Dendrogram analysis of ERIC-PCR fingerprints of VR *E. casseliflavus* isolates from different sources. The dendrogram was generated by the "Branch-and-bound" method using dollop program of PHYLIP 3.6 version

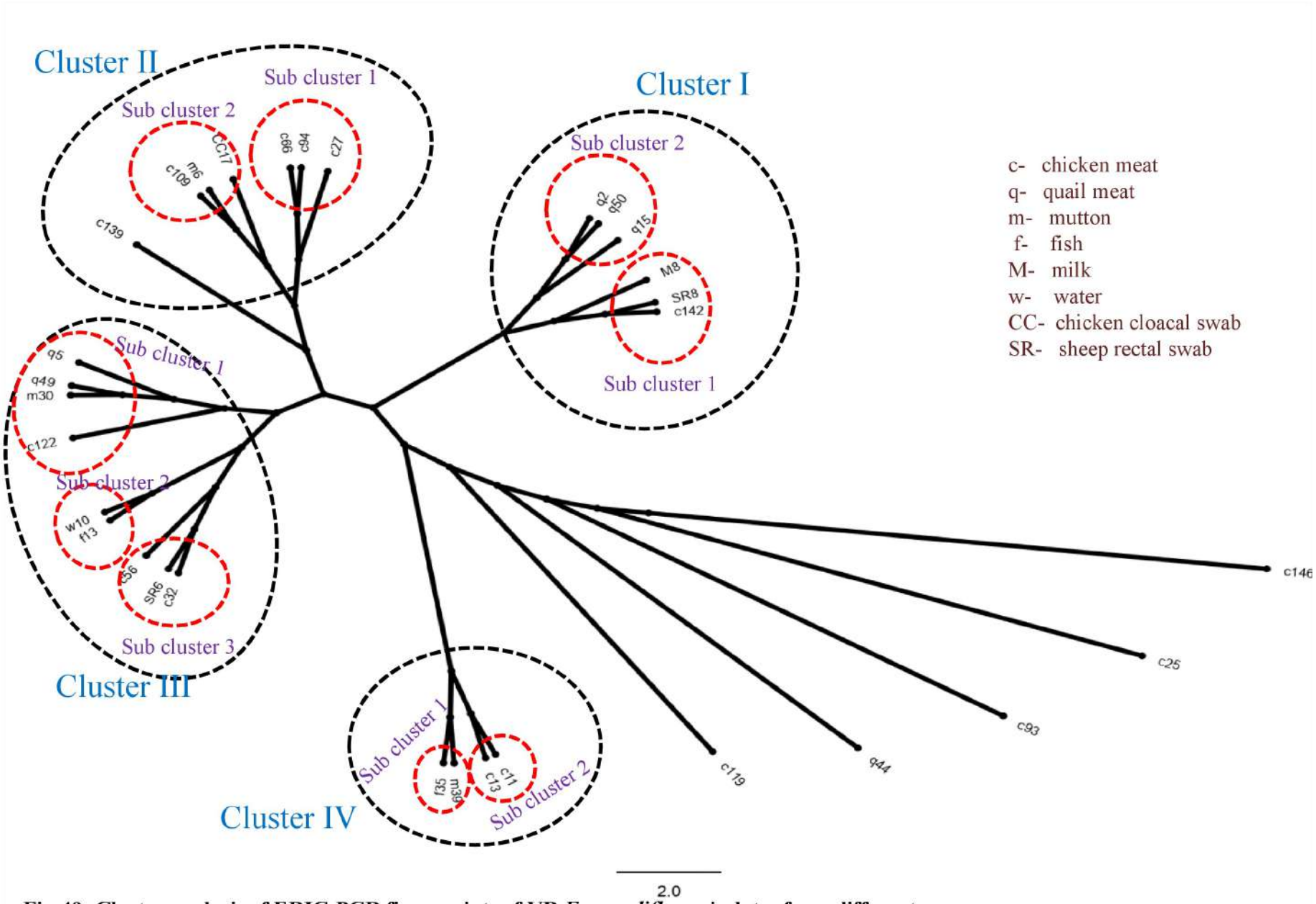
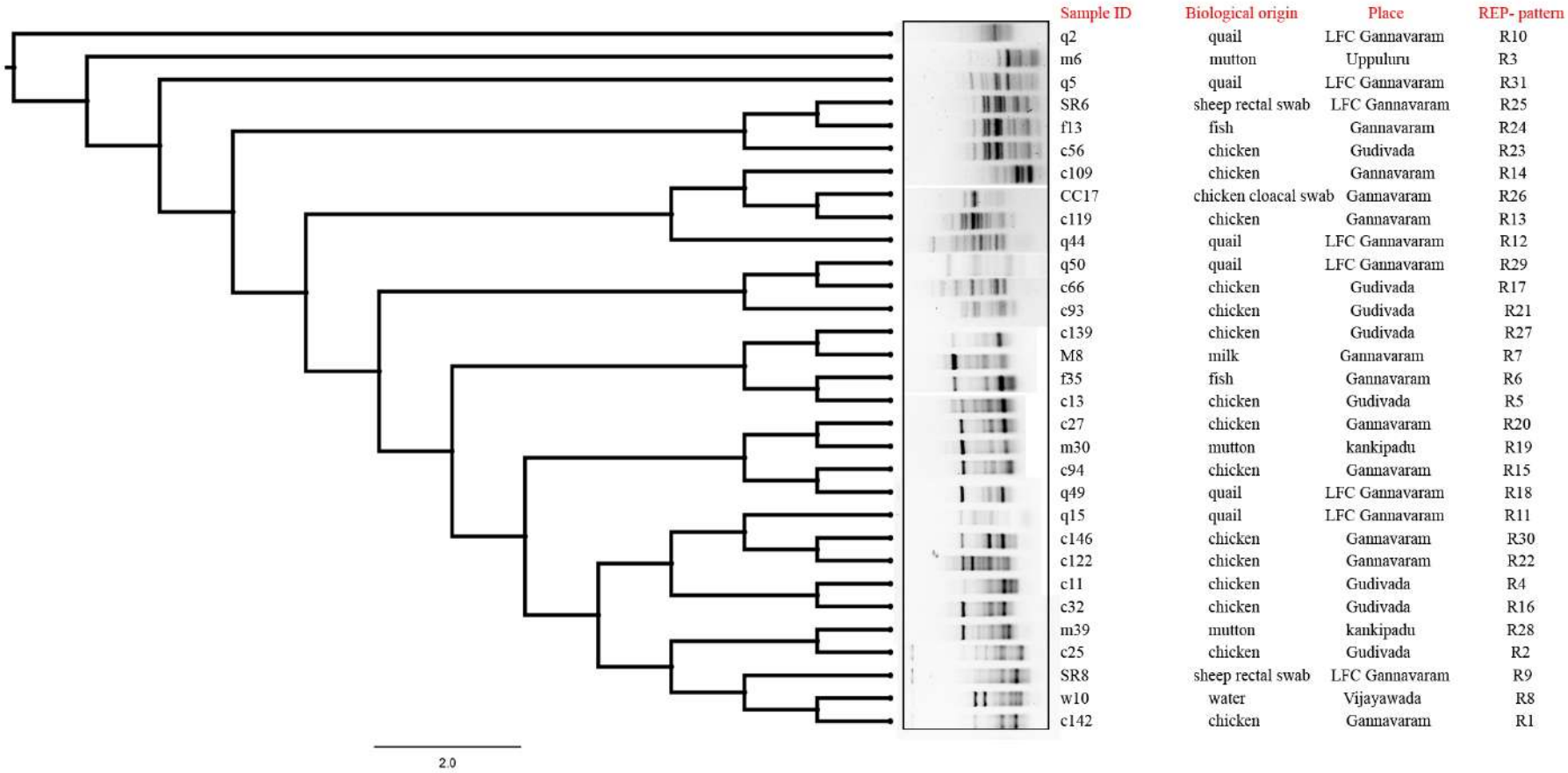


Fig-49: Cluster analysis of ERIC-PCR fingerprints of VR *E. casseliflavus* isolates from different sources.
An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm)



Sample ID	Biological origin	Place	REP- pattern
q2	quail	LFC Gannavaram	R10
m6	mutton	Uppuluru	R3
q5	quail	LFC Gannavaram	R31
SR6	sheep rectal swab	LFC Gannavaram	R25
f13	fish	Gannavaram	R24
c56	chicken	Gudivada	R23
c109	chicken	Gannavaram	R14
CC17	chicken cloacal swab	Gannavaram	R26
c119	chicken	Gannavaram	R13
q44	quail	LFC Gannavaram	R12
q50	quail	LFC Gannavaram	R29
c66	chicken	Gudivada	R17
c93	chicken	Gudivada	R21
c139	chicken	Gudivada	R27
M8	milk	Gannavaram	R7
f35	fish	Gannavaram	R6
c13	chicken	Gudivada	R5
c27	chicken	Gannavaram	R20
m30	mutton	kankipadu	R19
c94	chicken	Gannavaram	R15
q49	quail	LFC Gannavaram	R18
q15	quail	LFC Gannavaram	R11
c146	chicken	Gannavaram	R30
c122	chicken	Gannavaram	R22
c11	chicken	Gudivada	R4
c32	chicken	Gudivada	R16
m39	mutton	kankipadu	R28
c25	chicken	Gudivada	R2
SR8	sheep rectal swab	LFC Gannavaram	R9
w10	water	Vijayawada	R8
c142	chicken	Gannavaram	R1

Fig-50 Dendrogram analysis of REP-PCR fingerprints of VR *E. casseliflavus* isolates from different sources. The dendrogram was generated by the "Branch-and-bound" method using dollop program of PHYLIP 3.6 version

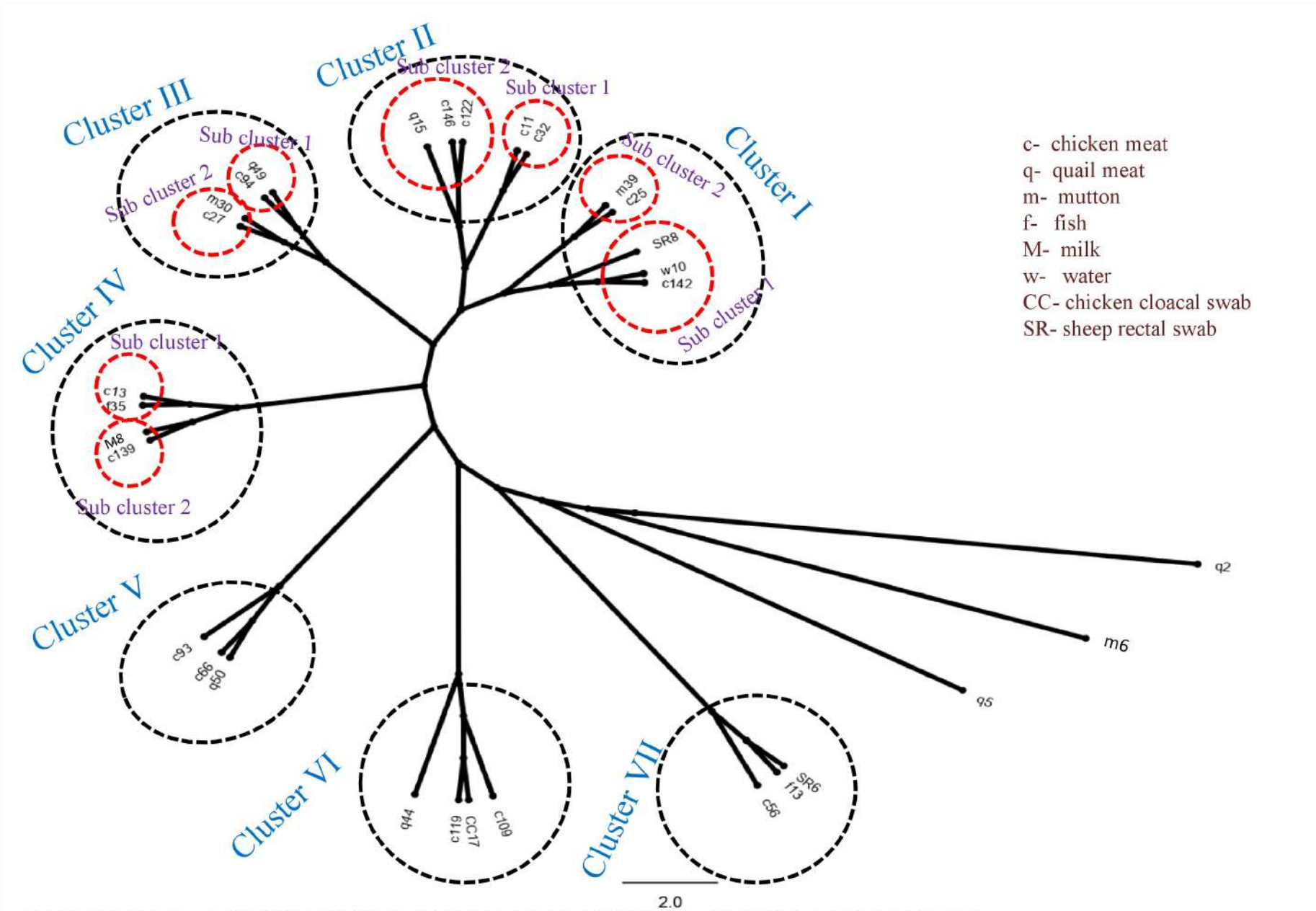


Fig-51: Cluster analysis of REP-PCR fingerprints of VR *E. casseliflavus* isolates from different sources.
An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm)

CHAPTER-IV

DISCUSSION

Enterococci are inhabitants of normal gut flora. Due to their ubiquitous nature, it determines their frequent finding in foods as contaminants. In addition, the notable resistance of enterococci to adverse environmental conditions explains their ability to colonise different ecological niches and their spreading within the food chain through contaminated animals and foods. Enterococci can also contaminate finished products, such as fermented foods and for this reason, their presence in many foods (such as processed meats, cheeses and fermented sausages) can only be limited but not completely eliminated using traditional processing technologies (Giraffa, 2002).

Antimicrobial resistant bacteria are nominated in the intestinal micro flora of animals, contaminate the foods of animal origin and transmit their resistance to other normal inhabitants of human gut, no matter if they are infectious or non-infectious. Resistance to antimicrobial agents may be an innate characteristic or it may be genetically acquired by gene transfer or gene exchange mechanisms (conjugation) that are susceptible to antimicrobials or via mutations. Enterococci are low grade pathogens but their intrinsic resistance to many antibiotics and their acquisition of resistance to antibiotics available for treatment in routine crafted them problematic. The virulence nature of *Enterococcus* spp. progressively increases due to resistance against commonly used antimicrobials (Sood *et al*., 2008).

Enterococcus spp. has come out as important pathogen associated with serious hospital acquired infections. They are stood up second most neighbour cause of nosocomial infections and the third most common cause of nosocomial bacteraemia. Although the role of non-human sources and reservoirs in the spread of *Enterococcus* strains is not clearly understood, animals, foods of animal origin and environmental

sources have been advocate to be sources for resistant isolates. It has been suggested that food animals may be the source of enterococci and resistance genes potential of transferring to humans through the food chain (Hershberger *et al*., 2005).

Taking above said hypotheses into attention, the present research study was carried out with an aim to elucidate the level of prevalence of *Enterococcus* spp. in different sources (foods of animal origin, animals, water and humans) with cultural isolation and identification methods supplemented with genetic molecular tools, to explore the antimicrobial profile, virulence marker profile and genetic diversity to serve as field level data.

Several media are available for isolation of *Enterococcus* spp. from different sources. Enterococci are easily recovered from the samples but isolates recovery varies with the media. There is no universal medium that will isolate all strains of enterococci. Since *E. faecalis* and *E. faecium* play a dominant role in food microbiology, KF-streptococcal agar was especially designed for this purpose (Kenner *et al.,* 1961). KF-streptococcal agar is a commercially available medium, which is used for the isolation and enumeration of enterococci from faeces, water and foods of animal origin (Mossel *et al.,* 1978). Many companies and organisations have approved the KF agar to be used for the quantitative enumeration of enterococci in water and non-dairy foods and KAA agar for dairy products. Considering all these data in the background, isolation protocol was standardized using these two media subjecting to pilot study and it was found that among different media, enrichment followed by plating on KF- streptococcal agar was found suitable for foods of animal origin and water as suggested by Messi *et al.* (2006) and for isolation of *Enterococcus* from dairy foods, KAA medium best suited as proposed by Gelsomino *et al*. (2002).

Biochemical characterization of *Enterococcus* spp. is generally not preferred owing to the biochemical inertness of the organism and inconsistency of results. More than that, several studies projected great deal of conflict among the biochemical tests of *Enterococcus* spp. In an attempt to avoid these ambiguities, apart from biochemical tests also molecular techniques were used to differentiate the organisms to the species level. Oligonucleotide primers targeting partial sequence of *sodA* (for genus) and *sodA* gene (species specific variation in nucleotide sequence of *sodA*) were used in PCR reaction to identify *Enterococcus* genus and 4 major species (*E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus*) of *Enterococcus*, respectively.

The samples from different sources were analysed for the presence of *Enterococcus* spp. by using 2 step procedure i.e. enrichment in BHI broth and selective plating on different *Enterococcus* selective agars (KF- streptococcal agar and KAA agar) and further confirmed by biochemical tests and molecular confirmation was done by using PCR targeting genus and species specific genes (Jahan *et al*., 2013 and Yilema *et al*., 2017).

Out of 780 samples comprising foods of animal origin and other sources analysed, 677 samples were found genotypically positive for genus *Enterococcus* with overall prevalence of 86.79%. The prevalence of the genus *Enterococcus* spp. in various samples was ranging from 100% each in sheep rectal swabs, pig rectal swabs, human diarrhoeic samples and water followed by 96.42% in pork, 94.44% in buffalo rectal swabs, 94.23% in carabeef, 92.85% in human stool samples, 91.80% in quail meat, 90.62% in chicken cloacal swabs, 89.02% in milk, 87.03% in fish, 85.54% in chicken, 76.25% in mutton, 55.55% in human urine samples and 55.00% in uterine discharges of cattle.

In the present study, overall prevalence of *Enterococcus* spp. (86.79%) was almost in agreement with the findings of Chandra and Garg (2006) who reported an overall prevalence of 94.6% in a sampling frame containing 37 meat samples, whereas higher the prevalence rate of 99% was reported by Hayes *et al*. (2003), with a sample frame containing 981 samples. In contrary, the present findings yielded a higher prevalence rate than the reports published by Kročko *et al.* (2011) with prevalence rate of 33.33%. The higher prevalence rate of *Enterococcus* in the present study may be due to difference in geographic location.

The findings of the present study revealed that 85.54% (148/173) prevalence of *Enterococcus* spp. in chicken meat was in concordance with findings of Quednau *et al.* (1998) who reported an overall prevalence of 77% in a sampling frame containing 211 samples of Danish chicken. In contrary, a much lower prevalence rate of 13% was reported by Kročko *et al.* (2011) in chicken samples in Slovakia where in higher prevalence rate of 96.14% was reported by Hayes *et al*. (2003) in retail chicken samples obtained from Maryland.

The findings from present study revealed 96.42% (54/56) prevalence of *Enterococcus* spp. in pork which was in agreement with findings of Hayes *et al*. (2003) and Quednau *et al.* (1998), who reported 93.28% in retail pork cuts and 90% in Danish pork, respectively. In contrary, low prevalence rate of 33.33% was reported by Pavia *et al.* (2000) from 24 pork samples collected from retail outlets of Catanzaro, Italy.

Overall prevalence of *Enterococcus* spp. in fish samples was 87.03% which was higher than the findings of Araujo *et al*. (2015) and Osman *et al*. (2016) who reported 42.2% and 8% in two different studies involving sampling frame of 200 samples of rainbow trout fish and 80 samples of tilapia, respectively.

Present study revealed 94.23% carabeef samples to be carrying *Enterococcus* spp. while lower rates of 83.12% and 76%, respectively were reported by Hayes *et al*. (2003) in retail beef cuts and Klein *et al.* (1998) from minced beef (275) in Berlin, Germany.

The prevalence of *Enterococcus* spp. in raw milk was 89.02%, which was lower than the findings of McAuley *et al*. (2015) and Pereira *et al.* (2017), who reported an overall prevalence of 96% in a sampling frame containing 211 samples of raw milk samples in Victoria and 96% in raw buffalo milk samples, respectively. In contrary, Hamzah and Kadim (2018) and Araya *et al*. (2005) reported only 31% and 38% of prevalence in two different studies in abroad involving similar sampling frame of raw milk samples.

Present study revealed 90.62% chicken cloacal swabs, 100% of sheep rectal swabs, 94.44% buffalo rectal swabs and 100% pig rectal swabs to be carrying *Enterococcus* spp. and the present findings were in agreement with the findings of Metiner *et al*. (2013), who showed 100% prevalence of Enterococcus spp. obtained from 69 faecal samples of pigs in turkey. Similarly Ünal *et al*. (2017) reported 97.91% prevalence of *Enterococcus* spp. in 240 chicken cloacal swabs in Turkey.

Prevalence of *Enterococcus* spp. observed from water samples in present study was 100% which was in concordance with findings of Asha Peter (2013) who reported an overall prevalence of 100% in a sampling frame containing 170 samples of water in Kerala. In contrary, Montiel *et al.* (2013) reported lower prevalence of 71% in lake water samples collected in Venezuela.

Overall prevalence of *Enterococcus* spp. in human stool samples was 92.85% and human diarrhoeic samples was 100% which was almost in agreement the findings

of Asha Peter (2013) who reported an overall prevalence of 100% in a sampling frame containing 200 samples of human faeces in Kerala. In contrary, the present findings yielded a lower prevalence rates compared to that of Kudaier (2007) and Biswas (2015) with 51.55% and 83.33%, respectively in two different studies involving almost similar sampling frame of human diarrhoeic samples. The variation in the prevalence rates can be attributed to geographical variations, sample size, seasonal variation and isolation approaches.

Present study revealed 55.55% of human urine samples to be carrying *Enterococcus* spp. whereas Desai *et al.* (2001) and Miskeen and Deodhar (2001) reported only 8.92% and 7.38% rates in two different studies conducted in India and Spain, respectively.

Out of 608 different species of *Enterococcus* under the study, *E. faecalis* was the most frequently isolated species from all different kinds of samples except carabeef and human stool samples.

In the present study, out of 677 *Enterococcus* isolates, 278 were *E. faecalis* (41.06%), 179 *E. faecium* (26.44%), 103 *E. gallinarum* (15.21%) and 48 *E. casseliflavus* (7.09%). Present study results were in agreement with Chingwaru *et al.* (2003), who reported high prevalence of *E. faecalis* (46.1%) followed by *E. faecium* (29.0%) and *E. casseliflavus* (7.6%) in foods of animal origin. They also reported lower rate of 7.4% *E. gallinarum* isolates. According to Chandra and Garg (2006) *E. faecalis* (73%) was the most prevalent species, followed by *E. gallinarum* (45.9%) and *E. raffinosus* (37.8%) in 37 meat samples of various species. They also reported less prevalence of other species including *E. faecium, E. durans, E. hirae, E. mundtii, E. solitarius, E. pseudoavium, E. dispar, E. cecoruum* and *E. avium*.

Among the *Enterococcus* isolates derived from chicken in the present study, *E. faecalis* was the dominant species with 41.89%, followed by 31.08% of *E. faecium*, 18.24% of *E. gallinarum* and 4.72% of *E. casseliflavus*. The results of the present study were contrary with the reports of Hayes *et al*. (2003) and Kročko *et al.* (2011), who showed variation in the prevalence rate of species with *E. faecium* and *E. gallinarum* were the dominant species in their studies, respectively.

Among the 54 *Enterococcus* isolates derived from the pork in present study, *E. faecalis* was identified as dominant species with 59.25%, followed by 16.66% of *E. faecium*. 5.55% of *E. casseliflavus* and 3.7% of *E. gallinarum*. Klein *et al.* (1998) and Knudtson and Hartman (1993) findings were in agreement with these findings and reported that *E. faecalis* was most prevalent than remaining *Enterococcus* spp. in pork samples examined during different stages in the slaughter processes as well as on pork products.

Out of 47 *Enterococcus* fish isolates, *E. faecalis* was evolved as dominant species with 57.44% followed by 29.78% of *E. gallinarum*, 8.51% of *E. faecium* and 4.25% of *E. casseliflavus*. These findings were in agreement with Savaşan *et al.* (2016), who identified *E. faecalis* (13%) to be the only species isolated whereas none of them gave a band for *E. faecium* and other *Enterococcus* species isolated from 200 fish samples in Turkey.

Out of 73 *Enterococcus* isolates isolated from raw milk samples of cattle, *E. faecalis* (38.35%) was evolved as dominant species followed by *E. faecium* (30.13%), *E. gallinarum* (15.06%) and *E. casseliflavus* (10.95%). This findings were supported by Chingwaru *et al.* (2003) where *E. faecalis* was the most predominant species (42.8%), followed by *E. faecium* (28.2%), *E. gallinarum* (7.9%), *E. avium* (6.2%), *E. durans* (2.5%), *E. hirae* (2.4%), *E. casseliflavus* (9.6%) and *E. mundtii* (0.4%) from 227 raw

and pasteurized milk samples. The same predominance pattern has also been reported by Citak *et al.* (2005). They reported that *E. faecalis* (54.2%) was the most frequently isolated species, followed by *E. faecium* (29%), *E. durans* (6.2%), *E. hirae/dispar* (5.0%), *E. gallinarum groups* (3.0%), *E. mundtii* (2.2%) and *E. raffinosus* (0.5%). The presence of *Enterococcus* in milk advocates improper sanitary conditions during the production and processing of milk. Wide range distribution of enterococci in the gastrointestinal tracts of milch animals and humans and the capability of the microorganisms to adapt to inauspicious environmental conditions are responsible for their frequent detection in milk. High prevalence rate of *Enterococci* in raw milk (89.02%) gives a reflection of poor sanitary conditions in the dairy farms and among the dairy workers.

Among 29 *Enterococcus* isolates recovered from chicken cloacal swabs, predominant species was *E. faecalis* (34.48%), followed by 24.13% of *E. gallinarum*, 13.79% of *E. faecium* and 6.89% of *E. casseliflavus*. Contrary to the present findings Ünal *et al*. (2017) reported *E. faecium* (60.43%) to be predominant followed by *E. faecalis* (33.62%), *E. casseliflavus* (3.42%) and *E. gallinarum* (2.56%) among broiler cloacal swab *Enterococcus* isolates in Turkey.

Out of 17 *Enterococcus* spp. isolated from buffalo rectal swabs, *E. faecalis* was evolved as dominant species with 58.82% followed by 29.41% of *E. faecium* and 5.88% of *E. gallinarum*. However Beukers *et al*. (2017) reported *E. hirae* (47.61%) was dominant species followed by *E. faecalis*, *E. faecium*, *E. casseliflavus*, *E. gallinarum* and *E. durans* each with prevalence rate of 4.76% obtained from bovine faeces.

Screening of 12 isolates recovered from pig rectal swabs both *E. faecalis* and *E. faecium* were predominant species (41.66% each) followed by *E. gallinarum* (8.33%). Present findings were almost in agreement with that of Metiner *et al*. (2013) who

showed *E. faecium* (68%) as predominant species followed by *E. faecalis* (21.7%), *E. gallinarum* (4.3%), *E. hirae* (1.4%), *E. casseliflavus* (1.4%), *E. cecorum* (1.4%) and *E. sulfurens* (1.4%) obtained from 69 pig faecal samples in Turkey.

Screening of 25 water samples yielded a prevalence rate of 100% in the present study. Out of 25 *Enterococcus* spp. recovered from water samples, predominant species was *E. faecalis* (48.00%). These findings were in coherent with the findings of Alipour *et al*. (2014) and Kimiran-Erdem *et al*. (2007) who reported the predominance of *E. faecalis* (68.6% in Babolsar and 96% in Istanbul, respectively) among the isolates from surface water. However, Enayati *et al*. (2015) reported *E. faecium* (37.46%) to be the predominant species followed by *E. galinarum* (34.92%), *E. hirea* (11.74%), *E. casselifelavus* (10.15%) and *E. mundeti* (5.71%) in water samples.

Enterococci are opportunistic pathogens and cause occasional infections. Habitually enterococci are a part of mixed flora commonly found in the gastrointestinal tract and it is difficult to differentiate colonization from true infection. Interactions among various bacteria have been demonstrated and several studies suggest that enterococci can act synergistically with other intestinal bacteria to enhance the rate of infection (Gao *et al*., 2018). *Enterococcus* species causing gastroenteritis in humans, *E. faecalis* is predominantly reported over remaining species of *Enterococcus* in many countries and from different samples. However variations exist, in terms of certain species (Biswas, 2015).

Out of 39 *Enterococcus* isolates recovered from human stool samples, *E. faecium* (41.02%) was identified as predominant species followed by *E. gallinarum* (28.20%), *E. faecalis* (20.51%) and *E. casseliflavus* (2.56%). Present study findings were greatly in agreement with the findings of Metallidis *et al*. (2006) where *E. faecium* was dominant (59.0%) followed by *E. gallinarum* (33.3%) and *E. casseliflavus* (7.7%).

In the present study, *E. faecalis* (41.66%) was the predominant species recovered from human diarrhoeic samples when compared to *E. faecium* (33.33%) and *E. gallinarum* (25.00%). This finding is supported by many reports such as Maschieto *et al*. (2004), who identified *E. faecalis* to be the prevalent species (53.6%) among intestinal strains. Shridhar and Dhanashree (2019) also reported high incidence rate of *E. faecalis* (58%) followed by *E. faecium* (42%) in their study.

Among 10 *Enterococcus* strains isolated from urine samples, 60% of strains were *E. faecalis* and remaining 4 isolates were not identified in the present study. The findings of Bertrand *et al*. (2000) and Miskeen and Deodhar (2002) were in agreement with the findings of the present study and they reported that 64% and 87% of isolates of *E. faecalis* from urinary tract infections of human, respectively.

In the present study, presence of phenotypic virulence factors among 608 *Enterococcus* isolates i.e. hemolysis of sheep RBC, slime layer formation, lipase activity, caseinase activity, biofilm formation, gelatinase, DNase activity and HA were detected in 312 (51.31%), 243 (39.96%), 47 (7.73%), 121 (19.90%), 236 (38.81%), 141 (23.19%), 37 (6.08%) and 87 (14.30%) of *Enterococcus* isolates, respectively. Characterization of phenotypic virulence factors in *E. faecalis* (278 isolates) revealed that slime layer formation was predominant 55.75% in *Enterococcus* isolates followed by hemolysis of sheep RBC (52.21%), biofilm formation (39.92%), gelatinase (31.29%), caseinase activity (23.02%), HA test (13.30%), lipase activity (12.23%) and DNase activity (5.03%). In a total 179 *E. faecium* isolates, predominantly hemolysis of sheep RBC observed in 44.13% isolates followed by biofilm formation (37.43%), slime layer formation (26.81%), gelatinase (24.58%), HA test (22.90%), caseinase activity (13.96%), DNase activity (11.17%) and lipase activity (5.02%). Out of 103 isolates of *E. gallinarum*, hemolysis of sheep RBC was observed in 68.93% isolates followed by

biofilm formation (39.80%), caseinase activity (23.30%), gelatinase (20.38%), slime layer formation and HA test (8.73% each) and DNase activity (2.91%). None of the *E. gallinarum* isolates showed lipase activity. Out of 48 *E. casseliflavus*, 64.58% isolates showed slime layer followed by hemolysis of sheep RBC (33.33%), biofilm formation (35.41%), gelatinase (18.75%), caseinase activity (16.66%) and lipase activity (8.33%). DNase activity and hemagglutination were not detected in any *E. casseliflavus* isolates of present study.

These findings were almost on par with Biswas (2015), who noticed that DNase activity and slime layer formation were common to all the three species (*E. faecalis*, *E. faecium* and *E. gallinarum*) being more common in *E. faecium* (17.5% in VRE isolates and 50.0% in non VRE isolates, respectively). She also reported absence of lipase and caseinase activity in *E. gallinarum* and adhesin molecule (responsible for biofilm formation) to be more common in *E. faecalis* strains. Gelatinase production was reported to be 25.0% each in all the three species. In contrary, she reported that low rate of hemolysis to sheep RBC (8.3% in *E. faecalis* and 12.5% in *E. faecium*). None of the *E. gallinarum* strains were hemolytic to sheep or human RBC. The present study results were supported by Trivedi *et al*. (2011), who identified phenotypic β-haemolytic activity in *E. faecalis* (29%) compared to *E. faecium* (10%). They also reported β-hemolytic activity in *E. mundtii* and *E. durans* of dairy origin and two *E. casseliflavus* strains of dairy and meat origin. Dworniczek *et al*. (2005) reported that none of *E. gallinarum*, *E. casseliflavus* and *E. durans* were haemolysin producers in their study.

Out of 608 different *Enterococcus* spp. under study, 236 isolates showed black colonies with crystalline consistency indicating the ability to form biofilm. The strength of biofilm formation was scored by MTP assay. Out of 236 biofilm forming *Enterococcus* isolates, 44 strong biofilm producers (18 *E. faecalis*, 13 *E. faecium*, 9 *E.*

gallinarum and 4 *E. casseliflavus*), 85 medium level biofilm producers (62 *E. faecalis*, 4 *E. faecium*, 14 *E. gallinarum* and 5 *E. casseliflavus*) and 107 weak biofilm producers (31 *E. faecalis*, 50 *E. faecium*, 18 *E. gallinarum* and 8 *E. casseliflavus*) were identified. Strong biofilm production (18.64%, 44/236) in present study is in perfect sync to the research findings of Peter *et al*. (2013) who showed that 13.46% of the enterococcal isolates obtained from different sources are strong producers of biofilm. Tsikrikonis *et al*. (2012) and Dworniczek *et al*. (2005) reported biofilm formation in 9.5% and 37% of *E. faecalis* and 34.4% and 20.4% of *E. faecium* isolates, respectively. Tsikrikonis *et al*. (2012) reported *E. faecalis* (18%) to be the foremost species capable of strong biofilm production followed by *E. faecium* isolates (11%) and it was absent in all other species of enterococci.

Characterization of virulence profile of food-borne microorganisms is ideal to favour virulotyping and thus elucidate the mechanisms of pathogenesis in the host. For this purpose, an investigation was carried out to detect the presence of seven virulence markers in the *Enterococcus* isolates, by a set of two m-PCR assays, which revealed the overall prevalence of *gelE* in 181 isolates (29.76%) followed by 180 *asa* (29.60%), 131 *hyl* (21.54%), 117 *ace* (19.24%), 101 *efaA* (16.61%) and 68 *cyl* (11.18%) genes. None of the *Enterococcus* isolates possessed *esp* gene, which is supposed to promote the primary attachment to biotic and abiotic surfaces and to be involved in hiding the protein from the immune system (ToledoArana *et al*., 2001). The detection rate of *asa* (responsible for aggregation) was in tune with the findings of Enayati *et al*. (2015) with 33.9% rate. They also reported lower prevalence rate of *cylA* (2.5%) and none of the isolates to be carrying *hyl* and *gelE* gene but higher rate of *esp* (84.7%) was reported. The *hyl* gene responsible for the hyaluronidase activity of enterococci was detected in 21.54% of the isolates and is almost in corroboration with the findings of Trivedi *et al*. (2011) who reported 19% of *hyl* in *Enterococcus* isolates. They also reported lower

prevalence rate of *gelE* (0.8%), *asa* (15%), *cylA* (2%) but higher rate of *efaA* (22%). Jahangiri *et al*. (2010) reported less prevalence rate of *hyl* (3.2%) and absence of genes *cylA*, *gelE* and *asa*.

In *E. faecalis*, the most frequently detected genes was *gelE* (30.57%) followed by *asa* (25.17%), *ace* (21.94%), *hyl* (20.64%), *efaA* (17.62) and *cylA* (11.15%). In contrary, the higher prevalence rates of virulence genes was reported by Golob *et al*. (2019) with predominance of *efaA* (95.8%) followed by ace (76.7%), *gelE* (65.8%), *asa* (31.7%) and *esp* (10.8%), respectively in red meat samples. They also reported less prevalence of *cylA* (5.00%) and absence of *hyl* gene in *E. faecalis* isolates obtained from red meat.

In *E. faecium*, the most frequently detected gene was *asa* (31.28%) followed by *gelE* (25.13%), *hyl* (22.34%), *ace* (16.75%), *efaA* (12.84%) and *cylA* (11.17%). These results were in tune with the results of Golob *et al*. (2019) for the genes *efaA* and *ace* (13.33% each) in *E. faecium* isolates obtained from human clinical samples. However, other genes evaluated by them showed higher frequencies i.e. *esp* (70%) and *hyl* (53.3%) whereas, *gelE*, *asa* and *cylA* (6.7% each) were recovered in low rate. In contrary to the present study Eputiene *et al*. (2012) and Golob *et al*. (2019) reported that none of the *E. faecium* isolates obtained from farm animal faecal swabs and retail cuts of red meat were carrying virulence genes.

In *E. gallinarum*, the most frequently detected genes were *gelE* and *asa* 32.03% each followed by *hyl* (23.30%), *efaA* (17.47%), *ace* (16.50%) and *cylA* (11.65%). These findings were in corboration with Biswas *et al*. (2016b), who reported 40% of *gelE*, 7.1% *cylA*, 15.3% *hyl* and 28.3% *asa* in clinical VRE isolates.

In *E. casseliflavus*, the most frequently detected genes were *asa* (43.75%) followed by *gelE* 18 (37.50%), *efaA* and *hyl* (22.91% each), *ace* (18.75%) and *cylA* (10.41%). Present study findings were on par with Trivedi *et al*. (2011), who reported *ace* (50.00%) predominantly followed by *esp* (33.33%), *hyl*, *asa* and *efaA* (16.66% each) in 75 *Enterococcus* isolates of meat origin.

All the Enterococcus isolates that phenotypically showing virulence factors may not necessarily always possess virulence genes genotypically and vice-versa. Earlier studies also reported similar findings (Biswas, 2015 and Versalovic *et al*., 1991).

Virulotyping is one of the new typing method being preferred in food-borne microorganisms and is considered expedient to genotyping (Timothy *et al*., 2008). Virulotyping of virulence genes carriying 128 *E. faecalis*, 83 *E. faecium*, 49 *E. gallinarum* and 25 *E. casseliflavus* isolates was attempted which yielded 38, 30, 20 and 15 virulotypes, respectively. This virulotyping method is directed at clustering of strains into virulent or avirulent based on the number and type of virulence genes present and it assists in understanding the epidemiology of *Entercoccus* infections in a better way which is otherwise termed complex. However, many researchers have opined that the concept of virulotyping is valid only when the definition of virulence genes and virulence assisted genes are clearly defined and demarcated. Still, further more studies need to be directed to validate the methods of virulotyping and demarcate virulence genes of *Enterococcus* from virulence associated genes. Similar virulotyping studies were done for *Campylobacter* spp. by Srinivas (2018) and he reported 5 and 7 virulotypes in *C. jejuni* and *C. coli*, respectively.

Emergence of antibiotic resistance in food-borne microorganisms has elevated the stakes on the global trade of foods of animal origin. As the world is creeping towards attaining self-sufficient food production to meet the needs of a dynamically

growing worldwide population, it turned out necessary to exercise surveillance on the antimicrobial resistance in food-borne microorganisms, which can be transferred through food and can have awful impacts on the health of the consumers. The emergence of multidrug resistant (MDR) strains of food-borne microorganisms pose a serious public health issue in developing countries (Tiwari and Dhama, 2014). Increasing antibiotic resistance in *Enterococcus* spp. recovered from animal sources has been reported worldwide (Guzman Prieto *et al*., 2016).

Keeping this in view, the present work was designed to study different antibiogram profiles of different Enterococcus species. A major fraction of the *Enterococcus* isolates showed sensitivity to ampicillin (90.78%) followed by piperacillin (81.90%), linezolid (78.94%), teicoplanin (78.61%), penicillin-G (76.48%), rifampicin (75.16%), vancomycin (74.67%), nitrofurantoin (74.67%), chloramphenicol (71.71%) and gentamicin (66.77%), tetracycline (48.19%), ciprofloxacin (47.86%), streptomycin (44.24%), erythromycin (19.73%), ceftazidime (6.74%) and polymixin-B (4.44%). All the *Enterococcus* isolates were resistant to at least one of the antibiotics tested. Higher resistance was observed for polymixin-B (95.55%) followed by ceftazidime (93.25%), erythromycin (77.63%), streptomycin (44.07%), tetracycline (34.70%), ciprofloxacin (31.08%), gentamicin (26.48%), penicillin-G (23.35%), rifampicin (21.21%), vancomycin (19.24%), chloramphenicol (16.77%), nitrofurantoin (14.80%), linezolid (11.01%), teicoplanin (10.52%), ampicillin (9.21%) and piperacillin (8.88%). Notable percentage of isolates were intermediately resistant against ciprofloxacin (21.05%) followed by tetracycline (17.26%), streptomycin (11.67%), chloramphenicol (11.51%), teicoplanin (10.85%), nitrofurantoin (10.36%), linezolid (10.03%), piperacillin (9.21%), gentamicin (6.74%), vancomycin (6.08%), rifampicin (3.61%), erythromycin (2.63%) and penicillin-G (0.16%).

The resistance towards streptomycin (44.07%) and gentamicin (26.48%) is a matter of concern, as it reduces the number of possible treatment options for enterococcal infections. Combination of ampicillin or pencillin with an aminoglycoside is generally indicated for the serious enterococcal infections such as endocarditis. The resistance rate for gentamicin in the present study was almost in tune with the findings of Krocko *et al.* (2011) who also reported a resistance rate of 25% towards gentamicin. However higher and lower rates have been demonstrated by Messi *et al.* (2006) and Kimiran-Erdem *et al*. (2007) who reported 92.8% and 2% of resistance towards aminoglycosides, respectively. Resistance towards streptomycin of the present study was almost in sync with the findings of Diarra *et al*. (2010) who reported a rate of 33.33% among *E. faecium* isolates. However higher levels of resistance towards streptomycin (88% and 97%) have been reported by Kimiran-Erdem *et al*. (2007) and Citak *et al.* (2005), respectively.

The present study depicted resistance levels of 77.63% and 21.21% towards erythromycin and rifampicin, respectively. The high level resistance to erythromycin in *Enterococcus* isolates is likely related to the wide use of these classes of antibiotics in husbandry activities, especially the extensive use of tylosin for growth promotion and treatment of diseases. Erythromycin resistance goes in line with a range of resistance reports ranging from 7% (Kimiran-Erdem *et al*. 2007) through 40% (Cariolato *et al*., 2008) to nearly similar 66.67% (Zou *et al*., 2011). The present study showed higher rates compared to that of 38% and 26.66% reported by Soares-Santos *et al*. (2015) and Valenzuela *et al*. (2008), respectively.

The present study revealed a resistance level of 31.08% towards ciprofloxacin, particularly higher in 88 *E. faecalis* and 54 *E. faecium*. Enterococci demonstrate low levels of intrinsic resistance to the quinolones, but can acquire high-level resistance

through several mechanisms. Mutations in the target genes, specifically *gyrA* and *parC*, have been described in *E. faecium* and *E. faecalis*, but are absent in *E. gallinarum* and *E. casseliflavus* (Werner *et al*., 2010, López *et al*., 2011 and Yasufuku *et al*., 2011). The present report was almost in tune with the findings of Kim *et al*. (2018) who reported a resistance rate of 24.92% towards ciprofloxacin. However lower and higher rates have been demonstrated by of Soares-Santos *et al*. (2015) and Valenzuela *et al*. (2008) who reported 0% and 66.66% resistance against ciprofloxacin, respectively.

Out of 608 isolates of *Enterococcus*, 581 (95.55%) and 567 (93.25%) and in *Enterococcus* isolates the present showed great level of resistance to polymixin-B and ceftazidime, respectively. Although the natural resistance of enterococci to cephalosporins is a well-known feature, but the molecular basis of this phenotype is not completely understood. A common observation is that intrinsic resistance is associated with a decrease in binding affinity of cephalosporins for the enterococcal PBPs, specifically PBP5 (Mainardi *et al*., 2000 and Arbeloa *et al*., 2004). Comparatively similar results were observed by Chandra (2003) with remarkable resistance to cephalosporins (42.7 to 100%) and polymyxin B (91.1%). Similar observations have been recorded by Klein *et al*. (1998) and Pavia *et al*. (2000) who reported resistance towards cephalosporins as 71.1% and 86%, respectively.

The present study depicted 34.17% of *Enterococcus* isolates showing resistance to tetracycline. Tetracycline is one of the most commonly used antimicrobial agents in India both for human and animal practices because of its cheapness and easy availability. The use of tetracycline in animal husbandry could lead to horizontal transfer of *tet* genes from poultry to human through the gut flora, especially enterococci (Ayeni *et al*., 2016). Among *Enterococcus* spp. *tet*(M) is the most frequently identified tetracycline-resistance gene and is most commonly located in the bacterial chromosome

and has been found to be associated with conjugative transposons related to the *Tn916/Tn1545* family (Rice, 1998 and Roberts, 2005). Comparatively low rate of tetracycline resistance has been reported by Klein *et al*. (1998) and Chingwaru *et al*. (2003) in enterococci isolated from minced beef and pork (20.1%) and isolates from milk, beef and chicken (l.9 to 26.3%), respectively. On the other side, Pavia *et al*. (2000) reported 84.4% of the *Enterococcus* isolates from meat to be tetracycline-resistant. Such variations in the resistance level to tetracycline might probably be due to the differences in the practice of using these drugs in animal husbandry.

While screening of 278 *E. faecalis* isolates, highest resistance was found against polymixin-B (94.60%) followed by ceftazidime (93.16%), erythromycin (84.17%), streptomycin (50.35%), tetracycline (41.36%), gentamicin (37.41%), ciprofloxacin (31.65%), rifampicin (30.21%), penicillin-G (23.38%), vancomycin (21.22%), chloramphenicol (20.86%), nitrofurantoin (18.70%), teicoplanin (12.58%) linezolid (10.43%), ampilcillin (10.07%) and piperacillin (8.99%). Antibiogram of 179 *E. faecium* isolates have shown highest resistance towards polymixin-B (95.53%) followed by ceftazidime (87.70%), erythromycin (79.88%), streptomycin (35.19%), tetracycline (29.05%), ciprofloxacin (30.16%), penicillin-G (21.22%), gentamicin (20.11%), rifampicin (15.64%), vancomycin (14.52%), nitrofurantoin (13.96%), chloramphenicol and linezolid (11.73% each), piperacillin (10.61%), ampilcillin (8.37%) and teicoplanin 5.02%).

The results of the present study were supported by Golob *et al*. (2019) where they reported *E. faecium* and *E. faecalis* isolates were more frequently resistant to erythromycin (82% and 77%, respectively) and streptomycin (60% and 83%) and less frequently resistant to ciprofloxacin (39% and 29%). They also noticed that chloramphenicol and gentamicin-resistance was more frequent among isolates of *E.*

faecalis, as compared to *E. faecium* (33% versus 11% and 40% versus 18%, respectively). Eputiene *et al*. (2012) reported a resistance level of 77% and 29% towards erythromycin and ciprofloxacin which were in tune with the present findings in *E. faecium*. They also reported similar resistance rates of erythromycin and ciprofloxacin with 82% and 39%, respectively in *E. faecium* isolates. Citak *et al.* (2005) noticed higher vancomycin and teicoplanin resistance rates of 48% and 52% in *E. faecalis* isolates, respectively and the corresponding values were 26% and 33% for *E. faecium*. In contrary, lower resistance rates of 7% to erythromycin, 6% to ampicillin, 3% to chloramphenicol, 2% to gentamicin and penicillin G were reported by Kimiran-Erdem *et al*. (2007). None of the strains in their study were resistant to vancomycin.

Screening of 103 *E. gallinarum* isolates towards antibiogram study, highest resistance was found against ceftazidime (100%) followed by polymixin-B (96.11%), erythromycin (63.10%), streptomycin (44.66%), tetracycline (37.86%), penicillin-G (32.03%), ciprofloxacin (30.09%), gentamicin (18.44%), chloramphenicol (16.5%), vancomycin (15.53%), linezolid (14.56%), rifampicin and teicoplanin (11.65% each), ampilcillin (10.67%), nitrofurantoin (6.79%) and piperacillin (2.91%). Similar findings of 100% and 89.5% were reported by Chandra (2003) against ceftazidime and polymyxin-B, respectively. Contrary to the present study, Oli *et al*. (2012) reported *E. gallinarum* isolates in their study to be sensitive to all antibiotics tested.

Out of 48 *E. casseliflavus* isolates, highest resistance was found against ceftazidime and polymixin-B (100% each) followed by erythromycin (62.5%), streptomycin (39.58%), ciprofloxacin and vancomycin (33.33% each), teicoplanin (8/48), piperacillin (14.58%) chloramphenicol, nitrofurantoin and penicillin-G (12.50% each), rifampicin and tetracycline (10.41% each), ampicillin, gentamicin and linezolid

(4.16% each). Biswas (2015) showed similar resistance rate against erythromycin (64.1%) in *E. casseliflavus* isolated from human faecal samples.

The commonly used method of control against *Enterococcus* infections are a synergistic combination of a cell wall mediating glycopeptide along with an aminoglycoside. The most commonly used aminoglycosides for this purpose is gentamicin. High level gentamicin resistance (HLGR) is mainly conferred by the gene *aac(6´)Ie-aph(2˝)Ia*, which inactivates gentamicin, kanamycin, tobramycin, netilmicin and amikacin. This gene is present in chromosomes or plasmids or associated with transposable elements which favour horizontal transfer of HLGR. High level resistance to gentamicin has been widely reported from various parts of the world.

Out of 271 *Enterococcus* isolates that were phenotypically resistant to aminoglycosides, 179 isolates were positive for HLAR genes and *aac(6´)Ie-aph(2˝)Ia* was the only gene detected in all isolates. In the present study, 29.44% (179/608) of different *Enterococcus* spp. were carrying HLAR gene. This finding was in agreement with the Salem-Bekhit *et al*. (2011) who also reported 20.24% of HLAR isolates among the 163 human clinical *Enterococcus* isolates. They also stated that along with *aac(6´)Ie-aph(2˝)Ia* other 4 genes *aph(2')-Ib* (7.36%), *aph(2')-Ic* (10.42%), *aph(2')-Id* (4.29%) and *aph(3')-IIIa* (14.11%) were also observed in human *Enterococcus* isolates.

Among the 179 *Enterococcus* HLAR isolates, *E. faecalis* (95, 53.07%) was the predominant followed by 44 *E. faecium* (24.58%), 30 *E. gallinarum* (16.75%) and 10 *E. casseliflavus* (5.58%) and they carried *aac(6´)Ie-aph(2˝)Ia* gene. Kaçmaz and Aksoy, (2005) reported that HLAR was found in 16% of *E. faecalis* and 88% of *E. faecium* for gentamicin and 35% and 44%, respectively for streptomycin. Lower resistance rates of 42.11% in samples of swine origin and 65% in human clinical samples were reported by

Mendiratta *et al.* (2008) and Jackson *et al.* (2005), respectively in two different studies in abroad with different sampling frame.

In the present study, 12% (3/25) of HLAR *Enterococcus* isolates originated from water showed resistance to gentamicin. Junco *et al*. (2001) notified 6.38% of HLAR isolates of water origin. The results of HLAR *Enterococcus* isolates in water indicate that HLAR are not limited to the clinical setting and may be recovered from a variety of aquatic environmental sources also.

In the present study, 43.58% (17/39) of HLAR enterococci were of human faecal origin. This finding almost similar with Padmasini *et al*. (2014) where they showed 38.2% isolates to be carrying *aac(6´)Ie-aph(2˝)Ia* gene. They also reported gene *aph(3')-IIIa* in 40.4% isolates. In our study, it was observed that some *Enterococcus* isolates with phenotypic resistance towards gentamicin and streptomycin antibiotics were not carrying the respective HLAR gene. Similar observations were made by Padmasini *et al*. (2014). This may be due to the expression of other HLAR genes that were not included in this study.

β-lactam antibiotics were considered as cornerstones in the therapeutic modalities in humans against bacterial infections and this status has been under threat by the emergence of β-lactamase producing micro-organisms. Though β-lactam antibiotics are the first line of treatment of human *Enterococcal* infections, it is necessary to understand the resistance mechanisms by which they operate. β-lactamase expression is considered as one of the major mechanisms of bacteria to inactivate β-lactam antibiotics which is originally described in staphylococci, the gene *blaZ* encodes a β-lactamase as part of an operon with *blaR1*, a trans membrane sensor and signal transducer, and *blaI*, a repressor gene (Hackbarth and Chambers, 1993). In contrast to staphylococci, *blaZ* in enterococci is expressed constitutively and at a much lower level,

resulting in a clinically significant inoculum effect. Thus, when inoculating bacteria at concentrations for routine susceptibility testing (generally 1×10^5 organisms per ml), the enterococci produce so little enzyme that they test susceptible, while at high inoculum, such as during infection, the presence of more enzyme leads to resistance (Murray, 2000).

Out of 608 *Enterococcus* isolates, 175 (33 ampicillin resistant only, 119 penicillin resistant only and 23 both ampicillin and penicillin resistant isolates) were found to be resistant to either penicillin or ampicillin or both were subjected to detection of *blaZ* gene, of which 127 (127/608, 20.88%) isolates were positive for *blaZ* gene. The present study findings were almost in accordance with Rizzotti *et al*. (2005) who reported 16.66% of *blaZ* positive *Enterococcus* isolates in the production chain of swine meat commodities. In contrary, lower *blaZ* rates of 3.33% in Argentine and 0.5% in Japanese retail ready-to-eat raw fish were reported by Lopardo *et al*. (1990) and Hammad *et al.* (2014), respectively. Di Cesare *et al*. (2014) reported higher rate of 45.4% of *blaZ* in environmental samples. In the present study, the *blaZ* gene was predominantly detected in *E. faecium* (48.81%), followed by *E. faecalis* (31.49%), *E. gallinarum* (13.38%) and *E. casseliflavus* (6.29%).

Vancomycin is considered as one of the antibiotic of last resort in treating the infections caused by Gram-positive bacteria. In general, enterococcus is considered as one of the safe microorganism associated with foods but, after acquiring antimicrobial resistance markers and virulence markers, they emerged as disease causing pathogens. VRE are the main sources of infections in humans and carriers of transferable vancomycin resistance markers to other bacteria (i.e. *Staphylococcus* spp.). A link between the use of avoparcin (probiotic action and prophylactic action) in poultry and swine farms and an increased occurrence of VRE in humans is well documented

especially in UK (Werner *et al*., 2008). Intrinsically VRE, such as *E. gallinarum* and *E. casseliflavus* acquire transferable vancomycin resistance markers, causing rare but significant opportunistic infections (Monticelli *et al*., 2018).

A total of 608 *Enterococcus* isolates of different species isolated from different sources were subjected for detection of vancomycin resistance by both phenotypic and genotypic methods. Out of 608 *Enterococcus* isolates, 117 (19.24%) isolates showed resistance to vancomycin by disc diffusion test (59 *E. faecalis*, 26 *E. faecium*, 16 *E. gallinarum* and 16 *E. casseliflavus*) and genotypically 125 (20.55%) were found to be VRE. Of 125 VRE positive genotypes, 21 were *E. faecalis* (3 *vanB*, 14 *vanC1* and 4 *vanC2*), 15 *E. faecium* (11 *vanB* and 4 *vanC2*), 58 *E. gallinarum* (52 *vanC1* and 6 *vanC2*) and 31 *E. casseliflavus* (3 *vanC1* and 28 *vanC2*) isolates. None of the isolates crried *vanA* gene. Out of 125 genotypically positive VRE isolates, the genes *vanB*, *vanC1* and *vanC2* were detected in 14 (11.20%), 69 (55.20%) and 42 (33.60%) *Enterococcus* isolates, respectively.

In present study, all *vanB* genotypes showed phenotypic resistance to vancomycin in disc diffusion test but *vanC1* and C2 genotypes mostly showed phenotypic sensitivity to vancomycin. The *vanA* gene mediated phenotype glycopeptide resistance is considered by acquired inducible high level resistance to both vancomycin and teicoplanin which has been notified in several *Enterococcus* spp. and in certain *Staphylococcus aureus* isolates that were showing phenotypic vancomycin resistance. *VanB* gene mediated phenotype glycopeptide resistance is associated with acquired inducible low to high level resistance to various concentrations of vancomycin but typically not to teicoplanin but few isolates with resistance also to teicoplanin have been describe (Arthur *et al.*, 1996a). *vanB* gene cluster was found predominantly in *E. faecalis* and *E. faecium* (Patel, 2005 and Taneja *et al.*, 2006b). *vanC* gene mediated

phenotype glycopeptide resistance is characterized by low-level vancomycin resistance and susceptibility to teicoplanin and has been described as an intrinsic property of *E. gallinarum* and *E. casseliflavus/ flavescens* (Billot-Klein *et al.,* 1994).

Out of 59 and 26 phenotypically positive VR *E. faecalis* and *E. faecium*, only 21 and 15 isolates were found to be carried VR genes, respectively. This expression of phenotypic VR and absence of VR genes of in the present study may be due to the presence of other VR genes which are not included in this study (Abadia-Patino e*t al*., 2004). Out of 58 and 31 VR genotypes of *E. gallinarum* and *E. casseliflavus* only 16 isolates of both were phenotypically resistance to vancomycin. This may be due to presence of *vanC* mediated intrinsic resistance mechanism (Billot-Klein *et al.,* 1994).

In the present study *vanC1* (55.2%) is the predominant VR gene followed by *vanC2* (33.60%) and *vanB* (11.20%) which were in agreement with the Xavier *et al*. (2006). They also reported *vanC1* as most prevalent vancomycin resistantce gene (13.0%) followed by *vanC2/3* (5.5%). Further they also reported that none of the isolates carried *vanA* or *vanB* genes of enterococci isolated from the chicken cloacal swab isolates obtained in Brazil.

Nishiyama *et al*. (2015) reported that 92% of river water *Enterococcus* isolates from Japan were carrying *vanC2/3*. Latha *et al*. (2016) reported an increased rate of *vanB* associated VRE isolates ranging from 22 to 100% from upstream to downstream in Gomati river water along the Lucknow city landscape.

In contrary to our findings Mac *et al.* (2003) reported *vanA* gene in 21 *Enterococcus* isolates from foods of animal origin and they could not detect *vanB* gene in any of the isolates, however majority of isolates possessed either *vanC1* or *vanC2.*

Peculiar findings in the present study include *E. faecalis* with 18 *vanC* genes (14 *vanC1* and 4 *vanC2*) and *E. faecium* with 4 *vanC2* genes. The detection of these *vanC* genes in *E. faecalis* and *E. faecium* is remarkable because they were thought acquire *vanC* genes by horizontal transfer from *E. gallinarum* and *E. casseliflavus*, natural inhabitants of the poultry gut in which *vanC* is intresic property. These findings were supported by Schwaiger *et al*. (2012), Moura *et al*. (2013) and Nishiyama *et al*. (2015), who also reported the presence of *vanC* genes in Enterococci isolates.

According to Ziech *et al*. (2016), organisms were considered as MDR when they do not show proneness to at least three classes of antibiotics. In the present study only VRE were considered for detection of MDR enterococci. In the present study, out of 125 VRE isolates, 95 (76.00%) isolates were showing multi-drug resistance. MDR was found in 19 (19/21, 90.47%) *E. faecalis*, 49 (49/58, 84.48%) *E. gallinarum*, 15 (15/15, 100%) *E. faecium* and 31 (31/31, 100%) *E. casseliflavus* isolates with average MAR index value 0.344. Very low MDR index values ranging from 0.06 to 0.19 were reported by Furtula *et al*. (2013). Higher index value of MAR in the present study may be attributed to the selection of VR resistance *Enterococcus* isolates only. MAR indexing of 21 VR *E. faecalis*, 15 VR *E. faecium*, 58 VR *E. gallinarum* and 31 VR *E. casseliflavus* yielded 19, 13, 50 and 30 MAR index groups, respectively.

These verdicts could be considered as thunderbolt for the future threats to come in terms of prevalence of antibiotic resistance prevalent in food associated pathogens and also necessitated the importance of continuous surveillance over the levels of antibiotic resistance especially in terms of MDR. MDR in foods of animal origin can be attributed to the use of prophylactic and growth promoting antibiotics at the farm level. These inexpert practices cannot be condoned as they come back to haunt the human healthcare system as a whole as they tend to make them ineffective.

Genetic fingerprinting by means of PCR based techniques like rep-PCR was undertaken to elucidate the genetic diversity present among the food borne pathogen isolates. In present study, rep-PCR was carried out using two different methods (ERIC-PCR and REP-PCR) which targets two different sets of repetitive elements. About 100ng of DNA of each isolate was genotyped by two typing methods (ERIC-PCR and REP-PCR) in triplicate.

Among rep-PCR typing methods, as most ERIC-PCR and REP-PCR methods suffer from reproducibility problem, the PCR reactions in the present study were standardized for their reproducibility by inclusion of DNA from *E. faecalis* (MTCC439) and *E. gallinarum* (MTCC7049). ERIC-PCR revealed genetic diversity between VRE species (*E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus*) with ERIC sequences found in all the *E. faecalis* isolates (4-9 distinct bands), *E. faecium* iaolates (3-11 distinct bands), *E. gallinarum* isolates (1-11 distinct bands) and *E. casseliflavus* isolates (2-12 distinct bands). REP-PCR revealed genetic diversity between the VRE species (*E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus*) with REP sequences found in all the *E. faecalis* isolates (3-12 distinct bands), *E. faecium* iaolates (3-14 distinct bands), *E. gallinarum* isolates (1-11 distinct bands) and *E. casseliflavus* isolates (3-12 distinct bands).

Among 125 VRE, greater degree of heterogeneity was observed among 124 VRE isolates (one *E. gallinarum* isolate did not show any bands for ERIC and REP-PCR) of different species from different sources as revealed by presence of 122 genotypes and 123 genotypes under ERIC and REP-PCR analysis, respectively. Nineteen different *E. faecalis*, 15 *E. faecium*, 57 *E. gallinarum* and 31 *E. casseliflavus* subtypes were differentiated by ERIC-PCR, whereas 21 different *E. faecalis*, 15 *E.*

faecium, 56 *E. gallinarum* and 31 *E. casseliflavus* subtypes were determined by REP-PCR, which revealed wide genetic diversity among the strains isolated.

Cluster analysis of ERIC-PCR profiles differentiated VR *E. faecalis* isolates from different sources into seven main clusters based on the genetic similarity cut-off value of 70%. Cluster I comprised of 3 isolates (c1, c9 and c17) of chicken samples obtained from Gannavaram, where c1 clustered separately from that of other two isolates (c17 and c9) recovered from same retail chicken shop. In cluster II, *E. faecalis* MTCC439 was closely clustered with isolate of mutton origin (m13) showing 90% similarity cut off. Clusters III and IV were having 2 sub clusters each with 2 isolates in each sub cluster. Cluster III comprised of four isolates of pork origin (p27, p31, p11 and p50) were recovered from pork processing unit, N.T.R.C.V.Sc. Gannavaram indicating the chances of cross contamination from equipment or lairage pen. Cluster V, VI and VII were having two isolates each. Cluster VI comprised of human diarrhoeic isolate (hd3) and chicken cloacal swab isolate (CC26) with same ERIC-PCR band pattern. Cluster VII consisted of two isolates of human diarrhoeic origin (hd2 and hd6) having similar ERIC band pattern. Isolates from chicken meat origin (c21 and c136) and fish origin (f11) unclustered separately indicating wide genetic diversity. However, cluster analysis of REP-PCR profiles grouped into four main clusters. Cluster I was again divided into 2 sub clusters having two isolates each (p17 and f11; f22 and c110, respectively) and p53 isolate was distantly related. Cluster II has three isolates (hd2, hd6 and p53) where p53 isolate was distantly related. Cluster III has two isolates (m13 and c9) whereas Clusters IV and V have four isolates each. In cluster IV, isolate from chicken (c21) was distantly separated from other three isolates (p31, c136 and p50). In cluster V, isolate from pork (p11) was separated from other three isolates (m12, c17 and CC26). Four isolates (c1, c39, hd3 and p27) and *E. faecalis* MTCC749 were found to be

unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates.

Cluster analysis of ERIC-PCR profiles differentiated VR *E. faecium* isolates from different sources into four main clusters for a similarity cut-off value of 70%. Cluster I again divided into 2 sub clusters, each sub cluster having two isolates. Cluster I (p14, c76, m10 and f39) and cluster II (p11, f14, c81 and c47) comprised isolates of meat origin. Within the cluster II, isolates p11 and c81 were clustered separately from that of other two isolates (f14 and c47). Cluster III comprised of two isolates recovered from samples of fish (f41 and f36) and 1 isolate recovered from water sample (w22) collected from Uppuluru showed closer genetic relatedness. It indicates the possibility of cross contamination between water body and fish available in the retail market from where the fish samples were collected. In cluster IV, one isolate recovered from water sample (w12) in Kankipadu and one isolate recovered from chicken meat (c84) in Gannavaram showed genetic closeness. However, cluster analysis of REP-PCR profiles differentiated VR *E. faecium* isolates from different sources into four main clusters for a similarity cut-off value of 70%. Cluster I and II have two isolates in each cluster (c76 and p11; c84 and f39, respectively) of animal meat origin. Cluster III and IV comprised of isolates recovered from water and foods of animal origin sharing closer genetic relatedness. Cluster III comprised of four isolates. In cluster IV, two isolates from water (w22) and fish (f36) obtained from Kankipadu showed the close genetic relatedness and similar sub clustering pattern observed in ERIC-PCR also. In cluster IV, c81 and f14 were closely related and w21 and c62 were separated from other two isolates. Isolates c47, f41 and p14 were unclustered indicating presence of wide genetic diversity. But they were clustered with other VR *E. faecium* isolates in ERIC-PCR dendrogram.

Cluster analysis of ERIC-PCR profiles differentiated VR *E. gallinarum* isolates from different sources into six main clusters for a similarity cut-off value of 70%. Cluster I was divided into four sub clusters. Isolates c65, c31, m5 and h17 were sub clustered of which c31 and m5 showed 90% similarity. Within cluster I, quail isolate (q46) was distantly isolated from the four sub clusters. Sub clusters 2 and 3 have two isolates each (CC31 and p36; BR9 and q31, respectively) and sub cluster 4 has three isolates (c3, q27 and m52). Cluster II comprised of three sub clusters where sub cluster 1 has two isolates (cb17 and M79), sub cluster 2 have three isolates (c101, M19 and *E. gallinarum* MTCC7049) whereas M19 and *E. gallinarum* MTCC7049 were closely related with >90% similarity cut off and sub cluster 3 consisted of four isolates (cb11, CC10, c79 and M76). Cluster III has five isolates (M4, q50, c115, CC20 and hd5) and they were divided into 2 sub clusters where, human diarrhoeic isolate (hd5) and chicken cloacal isolate (CC20) expressed close genetic relatedness. Cluster IV consisted of 11 isolates (M43, cb21, q22, h20, M24, cb5, m26, hd1, M77, c83 and PR6). Cluster IV comprised of three sub clusters where sub cluster 1 has M43, cb21 and q22, sub cluster 2 has six isolates (h20, M24, cb5, m26, hd1 and M77) where cb5 and m26 were closely related with >90% similarity cut off and sub cluster 3 has two isolates (c83 and PR6) Within Cluster IV, 3 sub-clusters were noticed. In cluster IV, human diarrhoeic (hd1) and human stool isolates (h20) were clustered together with isolates of meat and milk origin. Cluster V is the largest cluster consisting of 14 isolates and were grouped into five sub clusters where sub cluster 1 and 2 have two isolates each (c127 and CC13; c33 and m77, respectively), sub cluster 3 has c112, c99 and c153 isolates where c99 and c153 were closely related, sub cluster 4 has m25, c120 and w13 isolates of which c120 and m25 were closely related and sub cluster 5 has cb29, M3 and c159 isolates in which M3 and c159 are closely related. Within cluster V, chicken cloacal isolate (CC19) distantly away from other isolates that were sub clustered. Isolates, c153 and c99 of

subcluster 3 were obtained from same chicken retail shop in Gudiwada, but sampling interval in between 2 samples collected was 41 days. It indicates possibility of source of contamination from surrounding environment (*Enterococcus* spp. can withstand adverse environment with minimal nutrient requirement (Deibel and Hartman, 1984)). Cluster VI consisted of two isolates of chicken (c88) and chicken cloacal origin (CC8) which showed great genetic relatedness. Five isolates (w18, m21, q36, M39 and q24) were found to be unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates from different sources.

Dendrogram analysis of REP-PCR profiles discriminated VR *E. gallinarum* isolates into a 7 major clusters. Cluster I contained four isolates (c127, CC19, q22 and h17) where chicken cloacal isolate (CC19), showed closer genetic relation with isolate from quail (q22) origin. The clusters II and VII were the smallest clusters with three isolates each. Cluster II, human faecal isolate (h20) is distantly related with two isolates (cb21 and M79) that were sub clustered in the same cluster. Cluster III contained 10 isolates (m77, M77, w18, c101, CC13, c3, c36, c88, m25 and hd1). Within cluster III, 3 sub clusters were notified. Sub cluster 1 has hd1, m25, c88, q36 and c3 and sub cluster 2 and 3 had two isolates each (w18 and M77; c101 and m77). chicken cloacal isolate (CC13) was distantly related with other isolates in the cluster III. Cluster IV was the largest cluster with 16 isolates and comprised of 4 sub clusters where sub cluster 1 (q46, M24, cb5 and m5), 2 (m26, c33, m52 and q27) and 4 (M43, c31, w13 and p36) had four isolates each and sub cluster 3 has three isolates (c112, c120 and cb29). Within the cluster IV, chicken cloacal origin isolate (CC8) showed distant relation with other isolates of the sub clusters. Cluster V was divided into 4 sub clusters where sub clusters 2 (c153, c65 and c159), 3 (m21, c79 and hd5) and 4 (CC19, c99 and M3) had three isolates each and sub cluster 1 has two isolates (q31 and BR9). Within the cluster V, *E. gallinarum* MTCC7049 showed wide distance

with other isolates (4 sub clusters) within the cluster. Cluster VI contained 2 sub clusters where sub cluster 1 has q24, M4 and M76 and sub cluster 2 has PR6, c115 and CC31 isolates. Cluster VII contained three isolates (q50, cb17 and cb11) where quail isolate (q50) clustered separately from isolates of carabeef origin (cb17 and cb11). Isolates cb17 and cb11 showed close genetic proximity which were obtained from Kabela (Vijayawada) and it indicates possibility of contamination with similar *Enterococcus* strains occurred at slaughtering area. Four isolates (CC20, M39, c83 and M19) were found to be unclustered (UC) with other isolates.

Cluster analysis of ERIC-PCR profiles differentiated VR *E. casseliflavus* isolates from different sources into four main clusters for a similarity cut-off value of 70%. Cluster I comprised of 2 sub clusters where sub cluster 1 has c142, SR8 and M8 isolates and sub cluster 2 has three isolates of quail origin (q2, q50 and q15) showing close genetic relatedness. In cluster I, quail and sheep rectal isolate were obtained from LFC, Gannavram and remaining isolates were obtained from retail shops in Gannavarm. Cluster II comprised of 2 sub clusters where subcluster 1 has c27, c94 and c66 (isolates of chicken origin) and sub cluster 2 has CC17, m6 and c109. Within cluster II, isolate of chicken origin (c139), genetically far distant from other isolates of the cluster. Cluster III was divided into 3 sub clusters where sub cluster 1 has q5, q49, m30 and c122, where isolate of chicken origin (c122) arranged distantly from other three isolates, sub cluster 2 has w10 and f13 and sub cluster 3 has c56, SR6 and c32. Cluster IV again divided into two sub clusters and each with two isolates (c11 and c13; f35 and m39). Five isolates (c119, q44, c93, c25 and c146) were found to be unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates. However, cluster analysis of REP-PCR profiles differentiated VR *E. casseliflavus* isolates from different sources into seven main clusters for a similarity cut-off value of 70%. Clusters I was grouped into 2 sub clusters where sub cluster 1 has c142, w10 and

fm8 and sub cluster 2 has m39 and c25. Cluster II comprised of isolates recovered from samples of chicken (c11, c32, c122 and c146) and quail (q15). Within the cluster II, c32 and c11 were closely clustered which were obtained from same chicken retail shop in Gudiwada. It indicated the possible cross contamination either by handlers or equipment used for processing. In the same cluster II, chicken isolates (c146 and c122) collected from Gannavaram were also showing close proximity. Cluster III comprised of 2 sub clusters where sub cluster 1 has q49 and c94 and sub cluster 2 has m30 and c27. Similarly cluster IV again divided into 2 sub clusters where sub cluster 1 has c13 and f35 and sub cluster 2 has m8 and c139. Cluster V contained isolates of chicken (c93 and c66) and quail origin (q50) where c66 and q50 isolates showed great genetic relatedness. Cluster VI had 4 isolates (q44, c119, CC17 and c109) of which CC17 and c119 were showing close genetic association. Cluster VII has three isolates where isolates SR6 and f13 were showing close genetic relation (>90% similarity cut off value and c56 was distantly related with the other isolates f13 and SR6). Three isolates (q5, M6 and q2) were found to be unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates.

The discriminatory power of two typing methods i.e. ERIC-PCR and REP-PCR for *Enterococcus* isolates was found to be 0.9997 and 0.9999, respectively. The present study indicated both ERIC and REP-PCR to be highly suitable genotyping method, since discriminatory powers above 0.90 are considered highly significant (Hunter and Gaston, 1988). Thus rep-PCR (both ERIC and REP-PCR) fingerprinting methods can be used when large numbers of isolates are needed to be investigated. The present study reportage the genotyping and genetic diversity of VRE isolates recovered from animals, foods of animal origin, environment and humans, in India adds to the heterogeneity reports among Enterococcus species world-wide, supporting diversity among same species. The ERIC and rep-PCR analysis also indicated the genetic similarity among

diarrhoeic humans and meats of animals, which reveals the possibility of epidemiological relationship and evolutionary pattern between Enterococcus isolates of animal and human origin and its feasible zoonotic significance.

Present study results fall in line with Bedendo and Pignatari (2000), who reported discriminatory power of 0.9722 for 8 *E. faecium* isolates from Stanford University. They also reported that PCR based genetic diversity studies is of lower cost and is easier to perform than PFGE. However, PCR results are more difficult to analyze, since the presence of multiple weak bands in the PCR profiles makes it difficult to interpret the results. Similar results were also observed by Blanco *et al*. (2017), who conducted genetic diversity studies on one *E. hirae* and 67 *E. faecalis* strains by ERIC-PCR. They also reported that *E. fecalis* strains were clustered into five major groups and one strain was unclustered whereas *E. hirae* strain was distantly related to the rest of the strains.

The present study reported wide prevalence of different *Enterococcus* spp. from different sources and they were charaterized for their virulence and antibiogram profile and a wide genetic diversity prevailing over the VR *Enterococcus* spp. recovered from different sources. Among four species of *Enterococcus* isolated *E. faecalis* and *E. faecium* constituted the most frequently isolated species. The phenotypic virulence factors like hemolysis of sheep erythrocytes, DNase activity, lipase activity, caseniase activity, hemagglutination of RBC, slime layer formation and biofilm forming property and virulenece genes like *gelE*, *ace*, *efaA*, *asa*, *hyl* and *cylA* which were detected in the *Enterococcus* isolates from different sources give evidence of their potential to cause disease. HLAR, β-lactamase resistance, VRE and MDR were recorded among the isolates bearing great public health significance. Enterococci are well adapted for survival and persistence in a variety of adverse environments. These organisms are

though not highly toxigenic, highly invasive, highly infectious, these nevertheless, cause a substantial amount of human disease like UTI, bacteraemia, wound infections, intraabdominal infections and endocarditis. However, further studies are required in the direction of understanding the epidemiology and distribution of *Enterococcus* spp. Paradigm shift in the transfer of vancomycin resistance genes to resolve the debate over the development of vancomycin intermediate resistant MRSA (CDC listed new superbug) clear the air over the mechanisms of antibiotic resistance. In view of the above, consideration of enterococci as "generally recognized-as-safe" (GRAS) microorganisms in foods requires to be explored thoroughly. A constant vigilance is always required over the level of antibiotic resistance in different food-borne microorganisms which can make the life hard for the health-care specialists while treating patients. Thus, the concept of "One health" should be emphasized while undertaking future studies with an inter-disciplinary approach to get effective and rationale results.

CHAPTER-VI

SUMMARY

Genus *Enterococcus* is one of the major emerging antibiotic resistant organisms worldwide, predominantly transmitted by cross contamination. This study was intended to characterize *Enterococcus* spp., an organism with remarkable amounts of public health importance in terms of their prevalence in animals, foods of animal origin, environment and humans, based on cultural isolation as well as PCR detection (both at genus and species level), phenotypic virulence factors and virulence gene profiles, their antibiotic resistance patterns (phenotypic and genotypic methods) and genetic diversity.

A total of 780 samples were collected including 234 food samples from poultry (173 chicken and 61 quail); 324 food samples of animal origin like (56 raw pork, 80 mutton, 52 cara beef, 54 fish and 82 milk); 85 faecal swabs (32 chicken cloacal swabs, 18 buffalo rectal swabs, 23 sheep rectal swabs and 12 pig rectal swabs); 25 water samples; 40 uterine discharges of animals as well as 42 human stool samples, 12 human diarrhoeic stool and 18 human urine samples.

Out of 780 samples analysed, 681 (87.30%) samples were found to be positive for *Enterococcus* spp. by cultural methods. Among 681 isolates, 677 isolates were confirmed to be *Enterococcus* spp. by genus specific PCR and further subjecting these 677 isolates to species specific PCR revealed *E. faecalis* (41.06%) to be the predominant species followed by *E. faecium* (26.44%), *E. gallinarum* (15.21%) and *E. casseliflavus* (7.09%).

Of the 608 *Enterococcus* isolates, phenotypic virulence factors: hemolysis of sheep RBC, slime layer formation, lipase activity, caseinase activity, biofilm formation, gelatinase, DNase activity and HA test were detected in 312 (51.31%), 243 (39.96%),

47 (7.73%), 121 (19.90%), 236 (38.81%), 141 (23.19%), 37 (6.08%) and 87 (14.30%) *Enterococcus* isolates, respectively.

A total of 608 isolates of four major species of enterococci were subjected to preliminary identification of biofilm formation on Congo red agar. Out of 608 *Enterococcus* isolates, 236 isolates showed black colonies with crystalline consistency indicating the ability to form biofilm. Out of 236 biofilm forming *Enterococcus* isolates, 44 strong biofilm producers (18 *E. faecalis*, 13 *E. faecium*, 9 *E. gallinarum* and 4 *E. casseliflavus*), 85 medium level biofilm producers (62 *E. faecalis*, 4 *E. faecium*, 14 *E. gallinarum* and 5 *E. casseliflavus*) and 107 weak biofilm producers (31 *E. faecalis*, 50 *E. faecium*, 18 *E. gallinarum* and 8 *E. casseliflavus*) were identified by MTP assay.

Virulence gene profile of *Enterococcus* spp. revealed *gelE* in 181 (29.76%), *ace* in 117 (19.24%), *efaA* in 101 (16.61%), *asa* in 180 (29.60%), *hyl* in 131 (21.54%) and *cylA* in 68 (11.11%) *Enterococcus* isolates, respectively. None of the isolates showed *esp* gene. In the present study, all 608 *Enterococcus* isolates were subjected to virulotyping i.e. identification of combination of various virulence factor associated genes (*gelE*, *ace*, *efaA*, *asa*, *cylA* and *hyl*) where 38, 30, 21 and 15 virulotypes were identified for *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus*, respectively. A total of 323 *Enterococcus* isolates were not having any virulent marker.

Among 128 virulent *E. faecalis* isolates, maximum number of virulence genes were detected in 13 isolates belonging to virulotype Vf1. Lowest number of virulence associated genes i.e. one gene each was detected in 30 isolates belonging toVf33, Vf34, Vf35, Vf36, Vf37 and Vf38. The discriminatory power of virulotyping for *E. faecalis* was found to be 0.9541. Among 83 virulent *E. faecium* isolates, maximum number of virulence genes were detected in four isolates belonging to virulotype VTf1. Lowest number of virulence associated genes (1 gene each) was detected in 25 isolates

belonging to VTf26, VTf27, VTf28, VTf29 and VTf30. The discriminatory power of virulotyping for *E. faecium* was found to be 0.9521. Among 49 virulent *E. gallinarum* isolates, maximum number of virulence genes was detected in six isolates belonging to virulotype VTg1. Lowest number of virulence associated genes (1 gene each) was detected in 14 isolates belonging to VTg18, VTg19, VTg20 and VTg21. The discriminatory power of virulotyping for *E. gallinarum* was found to be 0.9371. Among 25 virulent *E. casseliflavus* isolates, maximum number of virulence genes were detected in two isolates belonging to virulotype VTc1. Lowest number of virulence associated genes (1 gene each) were detected in six isolates belonging toVTc14 and VTg15. The discriminatory power of virulotyping for *E. casseliflavus* was found to be 0.94.

All the 608 *Enterococcus* spp. isolates were subjected to antibiotic sensitivity test using 16 different most commonly used antibiotics in Veterinary medicine belonging to different classes. All *Enterococcus* isolates showed high rate of susceptibility to ampilcillin (90.78%) followed by piperacillin (81.90%), linezolid (78.94%), teicoplanin (78.61%), penicillin-G (76.48%), rifampicin (75.16%), vancomycin (74.67%), nitrofurantoin (74.67%), chloramphenicol (71.71%), gentamicin (66.77%), tetracycline (48.19%), ciprofloxacin (47.86%), streptomycin (44.24%), erythromycin (19.73%), ceftazidime (6.74%) and polymixin (4.44%). Notable percentage of isolates were intermediately resistant against ciprofloxacin (21.05%) followed by tetracycline (17.10%), streptomycin (11.67%), chloramphenicol (11.51%), teicoplanin (10.85%), nitrofurantoin (10.36%), linezolid (10.03%), piperacillin (9.21%), gentamicin (6.74%), vancomycin (6.08%), rifampicin (3.61%), erythromycin (2.63%) and penicillin-G (0.16%). Higher resistance was observed towards polymixin-B (95.55%) followed by ceftazidime (93.25%), erythromycin (77.63%), streptomycin (44.07%), tetracycline (34.70%), ciprofloxacin (31.08%), gentamicin (26.48%), penicillin-G (23.35%), rifampicin (21.21%), vancomycin (19.24%), chloramphenicol

(16.77%), nitrofurantoin (14.80%), linezolid (11.01%), teicoplanin (10.52%), ampicillin (9.21%) and piperacillin (8.88%).

Out of 278 *E. faecalis* isolates, highest resistance was found against polymixin-B (94.60%) followed by ceftazidime (93.16%), erythromycin (84.17%), streptomycin (50.35%), tetracycline (41.36%), gentamicin (37.41%), ciprofloxacin (31.65%), rifampicin (30.21%), penicillin-G (23.38%), vancomycin (21.22%), chloramphenicol (20.86%), nitrofurantoin (18.70%), teicoplanin (12.58%) linezolid (10.43%), ampilcillin (10.07%) and piperacillin (8.99%).

Among 179 *E. faecium* isolates, highest resistance was observed towards polymixin-B (95.53%) followed by ceftazidime (87.70%), erythromycin (79.88%), streptomycin (35.19%), tetracycline (29.05%), ciprofloxacin (30.16%), penicillin-G (21.22%), gentamicin (20.11%), rifampicin (15.64%), vancomycin (14.52%), nitrofurantoin (13.96%), chloramphenicol and linezolid (11.73% each), piperacillin (10.61%), ampilcillin (8.37%) and teicoplanin 5.02%).

Out of 103 *E. gallinarum* isolates, highest resistance was found against ceftazidime (100%) followed by polymixin-B (96.11%), erythromycin (63.10%), streptomycin (44.66%), tetracycline (37.86%), penicillin-G (32.03%), ciprofloxacin (30.09%), gentamicin (18.44%), chloramphenicol (16.5%), vancomycin (15.53%), linezolid (14.56%), rifampicin and teicoplanin (11.65% each), ampilcillin (10.67%), nitrofurantoin (6.79%) and piperacillin (2.91%).

Out of 48 *E. casseliflavus* isolates, highest resistance was found against polymixin-B and ceftazidime (100% each) followed by erythromycin (62.5%), streptomycin (39.58%), ciprofloxacin and vancomycin (33.33% each), teicoplanin (8/48), piperacillin (14.58%) chloramphenicol, nitrofurantoin and penicillin-G (12.50%

each), rifampicin and tetracycline (10.41% each), ampicillin, gentamicin and linezolid (4.16% each).

The *Enterococcus* isolates which exhibited phenotypic resistance for either gentamicin/kanamycin/streptomycin/amikacin were selected for molecular detection of HLAR genes. Out of 271 isolates that were phenotypically resistant to aminoglycosides, 179 (66.05%) isolates were positive for HLAR gene and *aac(6´)Ie-aph(2˝)Ia* was the only gene detected in the 179 isolates. Among the 608 *Enterococcus* isolates, 95 (15.62%) *E. faecalis* isolates, 44 *E. faecium* (7.23%), 30 *E. gallinarum* (4.93%) isolates and 10 (1.64%) *E. casseliflavus* were carrying *aac(6´)Ie-aph(2˝)Ia* gene.

Out of 608 *Enterococcus* isolates, 175 (33 ampicillin resistant, 119 penicillin resistant and 23 both ampicillin and penicillin resistant isolates) were found to be resistant to either penicillin or ampicillin or both and were subjected to detection of *blaZ* gene by PCR. Out of 175 isolates, 127 (72.57%) isolates were positive for *blaZ* gene. The *blaZ* gene was predominantly detected in *E. faecium* (62/127, 48.81%), followed by *E. faecalis* (40/127, 31.49%), *E. gallinarum* (17/127, 13.38%) and *E. casseliflavus* (8/127, 6.29%).

Out of 608 *Enterococcus* isolates, 117 (19.24%) isolates showed resistance to vancomycin by disc diffusion (59 *E. faecalis*, 26 *E. faecium*, 16 *E. gallinarum* and 16 *E. casseliflavus*) and genotypically 125 (20.55%) were found to be VRE. Out of 125 genotypically positive VRE isolates, the genes *VanB*, *VanC1* and *VanC2* were detected in 14 (11.20%), 69 (55.20%) and 42 (33.60%) *Enterococcus* isolates, respectively. Of 125 VRE positive genotypes, 21 were *E. faecalis* (3 *vanB*, 14 *vanC1* and 4 *vanC2*), 15 *E. faecium* (11 *vanB* and 4 *vanC2*), 58 *E. gallinarum* (52 *vanC1* and 6 *vanC2*) and 31 *E. casseliflavus* (3 *vanC1* and 28 *vanC2*) isolates. None of the isolates showed *vanA* gene.

Out of 125 (21 *E. faecalis*, 15 *E. faecium*, 58 *E. gallinarum* and 31 *E. casseliflavus*) VRE isolates, 114 (19 *E. faecalis*, 49 *E. gallinarum*, 15 *E. faecium* and 31 *E. casseliflavus*) isolates were MDR. MAR index values for VR *E. faecalis* were predominantly found to be above 0.2 except eight isolates. The average MAR index value was found to be 0.29. MAR index values for VR *E. faecium* were predominantly found to be above 0.2 except two isolates. The average MAR index value was found to be 0.345. MAR index values for VR *E. gallinarum* were predominantly found to be above 0.2 except 18 isolates. The average MAR index value was found to be 0.365. MAR index values for VR *E. casseliflavus* were predominantly found to be above 0.2 except two isolates. The average MAR index value was found to be 0.377. MAR indexing of 21 VR *E. faecalis*, 15 VR *E. faecium*, 58 VR *E. gallinarum* and 31 VR *E. casseliflavus* yielded 19, 13, 50 and 30 MAR index groups, respectively.

A total of 125 VR *Enterococcus* isolates (21 *E. faecalis*, 15 *E. faecium*, 58 *E. gallinarum* and 31 *E. casseliflavus*) including 2 MTCC strains (*E. faecalis* MTCC439 and *E. gallinarum* MTCC7049) were characterized further by two typing methods (ERIC and REP-PCR). Both ERIC and REP-PCR sequences were found to be present in 124 VR *Enterococcus* isolates except one isolate of *E. gallinarum*. The discriminatory power of the two typing methods i.e. ERIC-PCR and REP-PCR for *Enterococcus* isolates were found to be 0.9997 and 0.9999, respectively. The discriminatory powers above 0.90 are considered highly significant (Hunter and Gaston, 1988) and REP-PCR was found to be highly suitable genotyping method for genetic diversity studies of *Enterococcus* spp. Thus REP-PCR fingerprinting methods can be used when large numbers of isolates are needed to be investigated. Genotyping and genetic diversity of VRE isolates recovered from animals, foods of animal origin, environment and humans in India adds to the heterogeneity reports among Enterococcus species world- wide, supporting diversity among same species. The ERIC

and REP-PCR analysis also indicated the genetic similarity among diarrhoeic humans and meats of animals, which reveals the possibility of epidemiological relationship and evolutionary pattern between *Enterococcus* isolates of animal and human origin and its feasible zoonotic significance.

Key findings of the present study

1. Overall prevalence of *Enterococcus* spp. was observed to be 86.79% out of 780 samples. Highest prevalence was found in sheep rectal swabs, pig rectal swabs, human diarrhoeic samples and water (100% each), followed by 96.42% in pork, 94.44% in buffalo rectal swabs, 94.23% in carabeef, 92.85% in human stool samples, 91.80% in quail meat, 90.62% in chicken cloacal swabs, 89.02% in milk, 87.03% in fish, 85.54% in chicken, 76.25% in mutton, 55.55% in human urine samples and 55.00% in uterine discharges of cattle.
2. Detection of biofilm formation in 236 *Enterococcus* isolates by Congo red agar method. Out of 236 biofilm forming *Enterococcus* isolates, 44 strong biofilm producers (18 *E. faecalis*, 13 *E. faecium*, 9 *E. gallinarum* and 4 *E. casseliflavus*), 85 medium level biofilm producers (62 *E. faecalis*, 4 *E. faecium*, 14 *E. gallinarum* and 5 *E. casseliflavus*) and 107 weak biofilm producers (31 *E. faecalis*, 50 *E. faecium*, 18 *E. gallinarum* and 8 *E. casseliflavus*) were identified by MTP assay.
3. Detection of virulence genes in 285 isolates (46.87%) of the *Enterococcus* species and 323 *Enterococcus* isolates were found avirulent.
4. Resistance to 'first-line' antibiotics of human *Enterococcus* infection viz. pencillin (23.35%) and ampicillin (9.21%).
5. Resistance to last resort (serious infections) antibiotics of human *Enterococcus* infection viz. vancomycin (19.24%), teicoplanin (10.52%), gentamicin (26.48%) and streptomycin (44.07%)

6. Detection of HLAR gene, *aac(6´)Ie-aph(2˝)Ia* in 179 (29.44%) *Enterococcus* isolates.
7. Detection of *blaZ* gene in 127 (20.88%) *Enterococcus* isolates.
8. Alarming detection of VR markers in 129 (21.21%) *Enterococcus* species.
9. Among 129 VRE, 114 were found to be MDR. Among 95 MDR- VRE 19 were MDR- VR *E. faecalis* and 15 MDR- VR *E. faecium*, respectively, 49 MDR- VR *E. gallinarum* and 31 were MDR- VR *E. casseliflavus*.
10. Average MAR index values of VR *E. Faecalis*, VR *E. faecium*, VR *E. gallinarum* and VR *E. casseliflavus* were 0.29, 0.345, 0.365 and 0.377 for, respectively indicating exposure to high antibiotic environment.
11. Cluster analysis also revealed a great degree of homogeneity among some VRE isolates recovered from different sources. It implied at the chance of cross-contamination of foods of animal origin and thus can provide clue in understanding the complex epidemiology of this organism.

This study exteriorized the prevalence and distribution of *Enterococcus* at the species level in livestock, foods of animal origin, water and humans in Andra Pradesh, India. Cross-contamination (faecal contamination) is the chief mode of transmission to human beings, where it causes nosocomial infections in humans. The results obtained in the present study emphasized on the virulence factors profile, virulence gene profiles, antibiogram profiles (phenotypic and genotypic methods) and genetic diversity of VRE isolates of animal and human origin.

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APPENDIX-I

KF Streptococcal Agar Base

Ingredients	Gms / Litre
Peptone, special	10.000
Yeast extract	10.000
Sodium chloride	5.000
Sodium glycerophosphate	10.000
Maltose	20.000
Lactose	1.000
Sodium azide	0.400
Agar	20.000
Final pH (at 25°C)	7.2±0.2

Suspend 76.4 grams in 1000 ml distilled water. Add rehydrated contents of 1 vial of Bromo Cresol Purple (FD093). Heat to boiling to dissolve the medium completely. DO NOT AUTOCLAVE. Overheating will lower the pH and render the medium less productive. Cool to 50°C and aseptically add 10 ml of 1% 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) (FD057). Mix well and pour into sterile Petri plates.

Kanamycin Esculin Azide Agar

Casein enzymic hydrolysate	20.000
Yeast extract	5.000
Sodium chloride	5.000
Sodium citrate	1.000
Esculin	1.000
Ferric ammonium citrate	0.500
Sodium azide	0.150
Kanamycin sulphate	0.020
Agar	12.000
Final pH (at 25°C)	7.0±0.2

Suspend 44.67 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Dispense as desired.

Tryptone Soya Broth

Ingredients	Gms / Litre
Pancreatic digest of casein	17.000
Papaic digest of soyabean meal	3.000
Sodium chloride	5.000
Dextrose	2.500
Dibasic potassium phosphate	2.500
Final pH (at 25°C)	7.3±0.2

Suspend 30.0 grams in 1000 ml purified/ distilled water. Heat if necessary to dissolve the medium completely. Mix well and dispense in tubes or flasks as desired. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

DNase Test Agar

Ingredients	Gms / Litre
Tryptone	15.000
Soya peptone	5.000
Deoxyribonucleic acid (DNA)	2.000
Sodium chloride	5.000
Agar	15.000
Final pH (at 25°C)	7.3±0.2

Suspend 42 grams in 1000 ml distilled water. Heat with frequent agitation to dissolve the medium completely. Sterilize by autoclaving at 12 to 15 lbs pressure (118°C to 121°C) for 15 minutes. Cool to 45°C and pour into sterile petriplates. Add 0.1 gm Toluidine Blue (FD051) before sterilizing the medium or flood the plates with 0.1% Toluidine Blue (FD051) solution after incubation as desired.

BHI Agar (Brain Heart Infusion Agar)

Ingredients	Gms / Litre
HM infusion powder #	12.500
BHI powder	5.000
Proteose peptone	10.000
Dextrose (Glucose)	2.000

Sodium chloride	5.000
Disodium phosphate	2.500
Agar	15.000
Final pH (at 25°C)	7.4±0.2

Suspend 52.0 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates. If desired, 20 units Penicillin and 40 µg Streptomycin per ml of medium may be added to make the medium selective for fungi.

Egg Yolk Agar

Ingredients	**Gms / Litre**
Proteose peptone	40.000
Disodium phosphate	5.000
Monopotassium phosphate	1.000
Sodium chloride	2.000
Magnesium sulphate	0.100
Glucose	2.000
Hemin	0.005
Agar	25.000
Final pH (at 25°C)	7.6±0.2

Suspend 75.10 grams in 900 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense in 90 ml amounts and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and add 10 ml of sterile egg yolk emulsion (FD045) per 90 ml of medium. Mix well and pour into sterile Petri plates.

Ethidium bromide (10mg/mL)

Ethidium bromide (M/s. GeNei ^{TM}, Bengaluru)	100 mg
Double distilled water	10 mL

The suspension was stirred to ensure that the dye has dissolved. The container was then wrapped with aluminium foil and stored at 4˚C until further use.

APPENDIX II

Oxidase test

A loopful of culture was rubbed against the oxidase disc. Positive reaction was indicated by the development of violet colour within 5 - 10 sec, delayed reaction appeared within a minute and development of colour after one minute or no development of colour was considered as negative.

Catalase test

Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites. The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme. Small inoculum of bacterial isolate is mixed into hydrogen peroxide solution (3%) and is observed for the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. Catalase-positive bacteria include strict aerobes as well as facultative anaerobes. Catalase-negative bacteria may be anaerobes, or they may be facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor.

Hippurate hydrolysis test

Hippurate hydrolysis positive organisms can hydrolyze 1% aqueous sodium hippurate to produce glycine and sodium benzoate. Glycine is deaminated by the oxidizing agent ninhydrin which gets reduced and becomes purple. The test medium must contain only hippurate, since ninhydrin reacts with any free amino acids present. Suspend a loopful of growth from a suspect colony in 400 µl of a 1% sodium hippurate solution (care should be taken not to incorporate agar). Incubate at 37°C for 2 hours, and then slowly add 200 µl 3.5% ninhydrin solution to the side of the tube to form an overlay. Re-incubate at 37°C for 10 minutes and read the reaction. Dark purple/blue color appeared if reaction were positive while clear or grey color solution indicates negative reaction.

Voges Proskauer (VP) Test

Organisms such as members of the *Klebsiella-Enterobacter-Hafnia-Serratia* group produce **acetoin** as the chief end product of glucose metabolism and form smaller quantities of mixed acids. In the presence of atmospheric oxygen and 40% potassium hydroxide, **acetoin is converted to diacetyl**, and alpha-naphthol serves as a catalyst to bring out a red complex.

1. Inoculate a tube of MR/VP broth with a pure culture of the test organism.
2. Incubate for 24 hours at 35^{o}C
3. At the end of this time, aliquot 1 mL of broth to clean test tube.
4. Add 0.6mL of 5% α-naphthol, followed by 0.2 mL of 40% KOH.
5. Shake the tube gently to expose the medium to atmospheric oxygen and allow the tube to remain undisturbed for 10 to 15 minutes.

Preparation of 50 x TAE buffer

1) Weigh out 242 grams of Tris-base (MW = 121.14 g/mol) and dissolve in approximately 700 milliliters of deionized water.
2) Carefully add 57.1 milliliters of 100 % glacial acid (or acetic acid) and 100 milliliters of 0.5 M EDTA (pH 8.0).
3) Adjust the solution to a final volume of 1 liter. the pH of this buffer is not adjusted and should be about 8.5.
4) store stock solution at room temperature.

Preparation of 1 x TAE working solutuion

1) 20 ml 50 x TAE in 1000 ml of distill water.
2) final concentration in gel / running buffer: 40 mM Tris, 20 mM acetic acid, 1 mM EDTA

Phosphate Buffered saline (pH 7.4)

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Disodium hydrogen phosphate	1.44 g
Potassium dihydrogen phosphate	0.25 g
Double distilled water up to 1000.0 mL	