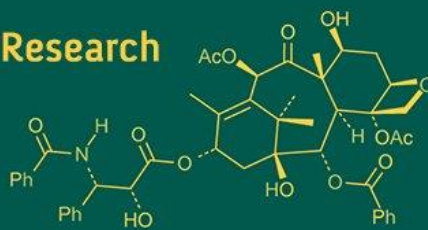


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693

ISSN Online: 2617-4707

NAAS Rating (2026): 5.29

IJABR 2026; 10(8): 53-58

www.biochemjournal.com

Received: 03-05-2026

Accepted: 10-06-2026

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Antibiotic resistance profiling of *Enterococcus* species isolated from animal-origin foods marketed in rural settings

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i8a.9441>

Abstract

The World Health Organization (WHO) has recognized *Enterococci* as a growing cause of nosocomial and antibiotic-resistant infections, posing a threat to public health. *Enterococci* contaminating animal-origin foods, such as meat, often carry drug-resistant and virulence genes. These species have developed resistance mechanisms to cope with adverse environmental conditions and are mostly transmitted through contaminated food. To better understand the prevalence and antibiotic resistance profiles, we characterized *Enterococci* isolated from locally marketed table eggs, raw chicken, mutton, and chevon. A total of 480 samples, 120 each of eggs, chicken, mutton, and chevon, were collected and bacteriologically cultured to detect *Enterococci*. Prevalence was recorded as raw chicken (42.50%), eggshell surface (40.00%), raw chevon (55.83%), and raw mutton (69.16%). The overall prevalence of *Enterococci* was 51.87%. PCR confirmation revealed a predominance of *E. faecium* over *E. faecalis*. The *hyl* gene was present in 8.08% of *E. faecium*. *Enterococci* were multidrug-resistant, showing greater resistance to tetracycline, vancomycin, erythromycin, and rifampin. They were mostly sensitive to gentamicin, amoxicillin-clavulanic acid, chloramphenicol, and ciprofloxacin. This study highlights the presence of antibiotic-resistant *Enterococcus* species in raw meat and on eggshell surfaces. The AMR monitoring program needs to be strengthened, given the importance of *Enterococci* in raw animal-origin foods.

Keywords: Antibiotic resistance, *Enterococcus*, animal-origin foods

Introduction

Enterococcus species are Gram-positive bacteria and are among the ESKAPE organisms highlighted by the World Health Organization. There are over 73 known *Enterococcus* species, and two species of public health importance are *E. faecalis* and *E. faecium* (Fahim *et al.*, 2025) [6]. *Enterococci* are highly resilient and present as commensals in the gut of birds, animals, and humans (Said *et al.*, 2025) [15]. They are primarily transmitted through the fecal-oral route and excreted in human and animal fecal matter. This bacterium is a significant cause of nosocomial infections, often due to virulent, antibiotic-resistant strains. The role of livestock and poultry in harboring antibiotic-resistant *Enterococci* and in the fecal excretion of these organisms, as well as their prevalence in raw animal-origin foods in the Indian context, is not well documented, except for a few studies (Singh and Garg, 2005; Parmar *et al.*, 2022; Kumar *et al.*, 2024) [17,11,14]. *Enterococci* are among the priority bacteria being monitored for the development and spread of antimicrobial resistance (AMR) in developed countries. *Enterococci* can colonize different ecological niches and could spread through contaminated animal-origin foods within the food chain. They are intrinsically resistant to several antibiotics and also can acquire and transfer AMR determinants to other species (Iweriebor *et al.*, 2015) [9]. Fresh meat is preferred in India; unhygienic dressing, cutting, and handling operations may cross-contaminate it. Thus, it is of paramount concern to study *Enterococcus* species for their prevalence and antibiotic resistance. This study was undertaken to determine the prevalence of *Enterococci* in retail meat and eggs commonly sold in the local markets.

2. Materials and Methods

2.1 Sampling

In this cross-sectional study, the prevalence of *Enterococcus* species was estimated from randomly collected samples from a local market. The sample size was calculated using Epitools (<https://epitools.ausvet.com.au/>) assuming a prevalence of 40%, a desired precision of 0.05, and a 95% Confidence Interval (CI). A total of 480 samples of raw chicken, mutton, chevon, and table eggs were collected from local chicken and meat shops. The distribution of samples was kept uniform, i.e., 120 samples each of chicken, mutton, chevon, and table eggs.

2.2 Isolation and identification of *Enterococcus* species

Samples were initially diluted to a 1:10 ratio in peptone water, and 1 mL of the diluted sample was enriched in azide dextrose broth (HiMedia) at 37 °C for 24 hr. Enriched samples were plated on bile esculin agar (HiMedia) and incubated at 37 °C for 24 hr. Presumptive identification of *Enterococci* was done based on colony characteristics, Gram's staining, catalase, oxidase, sorbitol fermentation, and bile esculin hydrolysis. The pure isolated colonies of *Enterococci* were further sub-cultured on the selective medium (bile esculin agar) and confirmed by polymerase chain reaction (PCR).

2.3 Molecular detection

A pure bacterial colony was subjected to DNA extraction using the boiling-and-snap-chilling method. The snap-chilled samples were centrifuged at 10000 g for five minutes (Sorvall ST 8, Thermo Scientific), and the supernatant was used as a DNA template. The DNA concentration was examined using a NanoDrop Lite spectrophotometer (Thermo Scientific). Bacterial DNA was further analyzed by PCR to confirm the presence of *Enterococci* at the genus and species levels. The oligonucleotide sequences used and the PCR protocol used in the study were as described earlier (Kariyama *et al.*, 2000) [10]. The multiplex PCR was performed in a 30 µl reaction containing 12.5 µl 2x PCR Master Mix (Takara), 0.5 µl (10 pmol/µl) of forward and reverse primers of *E. faecalis*, 0.25 µl (10 pmol/µl) of forward and reverse primers of 16 rDNA and 0.125 µl (10 pmol/µl) of forward and reverse primers of *E. faecalis*, 13.25 µl nuclease-free water, and 2.5 µl DNA. PCR conditions were set as initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 1 min, and final extension was performed at 72 °C for 10 min and holding at 4 °C (Applied Biosystems VeritiPro). The *hyl* gene was detected as per the method described by Vankerckhoven *et al.* (2004) [19]. The uniplex PCR was performed in a 25 µl reaction containing 12.5 µl 2x PCR Master Mix (Takara), 1 µl (10 pmol/µl) of forward and reverse primers, 5.5 µl nuclease-free water, and 5 µl DNA. PCR conditions were set as initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min, with a final hold at 4 °C. The PCR products were run on a 1.5% agarose gel stained with ethidium bromide, and images were captured using the gel documentation system (IBright CL750, Thermo Scientific). Details of PCR primers are depicted in Table 1.

2.4 Antibigram

Antibiotic sensitivity and resistance profiling of isolated *Enterococci* was performed by the disk diffusion method described previously (Bauer *et al.*, 1966), and results were interpreted according to the CLSI (2020) [5] guidelines and the manufacturer's instructions (HiMedia). *Enterococcus* species were studied using 10 antibiotics from different classes: aminopenicillins & β-lactams, amphenicol, fluoroquinolones, lincosamides, tetracyclines, macrolides, aminoglycosides, and rifamycins. The antibiotic discs procured from HiMedia Lab. Pvt. Ltd., Mumbai, India, used in this study were amoxycylav (20/10 µg), ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), doxycycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), rifampicin (15 µg), and tetracycline (30 µg). Pure colonies of *Enterococci* were first overnight enriched in Brain Heart Infusion (BHI) broth at 37 °C, and the enriched cultures were then spread onto Mueller-Hinton agar (MHA). Antibiotic discs were aseptically placed on the plates and incubated overnight at 37 °C.

2.5 Statistical analysis

The prevalence of *Enterococci* in food samples was calculated, and a significant association between *Enterococci* and antibiotic resistance and sensitivity was assessed using the Chi-square (χ^2) test (*p*-value), at a 95% confidence level.

3. Results

In this study, 480 samples were collected, and the prevalence of *Enterococcus* species was estimated. A total of 249 *Enterococci* (51 chicken, 48 eggshell, 67 chevon, 83 mutton) were isolated and identified using cultural and molecular methods. The overall prevalence was 51.87%, and the distribution across samples was as follows: chicken (42.50%), eggs (40%), chevon (55.83%), and mutton (69.16%). Multiplex PCR targeting 16S rRNA, *ddl E. faecalis*, and *ddl E. faecium* genes revealed the presence of *E. faecalis* (28.57%) and *E. faecium* (82.14%) in eggshell-surface-origin isolates. Other isolates from chicken, chevon, and mutton were *Enterococci* harboring the 16S rRNA gene; however, they may belong to species other than *E. faecalis* and *E. faecium* (Fig 1). The *hyl* gene was present in 16.66%, 14.92%, and 28.91% of isolates from eggshell surface, chevon, and mutton, respectively (Fig 2). The antibiotic susceptibilities of *Enterococcus* species isolated from chicken, eggshell surface, chevon, and mutton are shown in Tables 2, 3, 4 & 5. It was observed that *Enterococci* isolated from meat and chicken are multidrug-resistant, with greater resistance to clindamycin, rifampicin, erythromycin, and tetracycline. However, chicken- and egg-origin *Enterococci* showed sensitivity to antibiotics, viz., amoxicillin-clavulanate, ampicillin, ciprofloxacin, chloramphenicol, and gentamicin. The chevon and mutton origin isolates were mostly sensitive to amoxicillin-clavulanate, chloramphenicol, ciprofloxacin, and gentamicin.

4. Discussion

In the current study, the prevalence of *Enterococcus* species in animal-origin raw foods, namely chicken chevon, mutton, and table eggs, was estimated. The recorded prevalence is towards the higher side, indicating unhygienic processing and cross-contamination of raw meat. A study from Anand, Gujarat, recorded an overall prevalence of 56% for

Enterococci in chicken meat collected from retail chicken shops, and over 77% of *Enterococci* were positive for the *ddl* *E. faecalis* gene (Parmar *et al.*, 2022) ^[14]. The present findings are consistent with these observations on the overall prevalence of *Enterococci*. Studies from Hisar, Haryana, documented an *E. faecalis* prevalence exceeding 79% in animal products such as chicken, chevon, and milk (Chandra and Garg, 2005; Singh and Garg, 2005) ^[4,17], and confirmed the ubiquitous occurrence of *Enterococci* in foods of animal origin. Another study from Andhra Pradesh also detected *Enterococci* on pig farms and in retail pork and confirmed that the species were *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. casseliflavus* (Kumar *et al.*, 2024) ^[11]. This bacterium is not very extensively studied in India, and there are no references available from the state of Maharashtra. However, several studies worldwide have reported its prevalence in animal-derived foods. *Enterococci* are the natural inhabitants of the gastrointestinal tract (GIT) of humans and animals. *Enterococcus* species, namely *E. faecalis*, *E. faecium*, and *E. durans*, are capable of causing human infections, and *E. faecalis* and *E. faecium* are the most abundant species in human feces. However, animal GIT also represents a predominant reservoir for the different species of *Enterococci* (Lebreton *et al.*, 2014) ^[12]. During slaughter and dressing operations on meat animals, *Enterococci*, along with other gastrointestinal microbes, may contaminate meat. The species prevalent in meat animals include *E. hirae*, *E. faecium*, *E. faecalis*, *E. casseliflavus*, *E. mundtii*, *E. durans*, and *E. gilvus*. A study from Turkey reported the prevalence of *Enterococcus* species in healthy sheep and goat carcasses at 60% and 36.6%, respectively (Cebeci, 2024) ^[3]. In the current study, we have also recorded a higher prevalence of *Enterococci* in raw sheep and goat meat samples collected from retail meat shops. Consistent with our observations, Amuasi *et al.* (2023) ^[1] also reported a high prevalence of *Enterococci* in chevon and mutton samples collected from slaughterhouses in southern Ghana. A study conducted in the United States of America revealed that *E. faecalis* and *E. faecium* were the predominant species in chicken meat, regardless of antibiotic use (Manson *et al.*, 2019) ^[13]. Predominance of *E. faecium* in the chicken breast (79%) was observed in the study from Maryland (Hayes *et al.*, 2003) ^[8]. Over 79% of prepacked chicken samples collected from supermarkets in Turkey were contaminated with *Enterococci* (Sanlibaba *et al.*, 2018) ^[16].

Virulent *Enterococcus* species may harbor specific genetic sequences that enable them to evade the host immune system. Some of the predominant virulence genes in *Enterococci* are *gelE*, *esp*, *cylA*, and *hyl*. We have studied *Enterococci* for the presence of the *hyl* gene, which encodes

hyaluronidase, an enzyme that degrades hyaluronic acid in connective tissue. It was present in 16.66%, 14.92%, and 28.91% of isolates from eggshell surface, chevon, and mutton, respectively. The *hyl* gene is predominantly present in clinically important *Enterococci* and is mostly detected in human-origin isolates. In contrast to our observations, the *hyl* gene could not be detected in the *Enterococci* isolated from red meat in a study from Slovenia (Golob *et al.*, 2019) ^[7].

The antibiotic susceptibilities of *Enterococcus* species isolated from chicken, eggshell surface, chevon, and mutton are shown in Tables 2, 3, 4 & 5. It was observed that *Enterococci* isolated from meat and chicken are multidrug-resistant, with greater resistance to clindamycin, rifampicin, erythromycin, and tetracycline. However, chicken- and egg-origin *Enterococci* showed sensitivity to antibiotics, viz., amoxicillin-clavulanate, ampicillin, ciprofloxacin, chloramphenicol, and gentamicin. The chevon and mutton origin isolates were mostly sensitive to amoxicillin-clavulanate, chloramphenicol, ciprofloxacin, and gentamicin. As observed in the current study, *Enterococci* resistant to erythromycin and tetracycline and sensitive to gentamicin and chloramphenicol were also documented in India from *Enterococci* isolated from chicken meat (Parmar *et al.*, 2022) ^[14]. *Enterococci* are intrinsically resistant to cephalosporins and exhibit reduced susceptibility to penicillins due to low-affinity penicillin-binding proteins (Torres *et al.*, 2018) ^[18]. *Enterococci* are highly resistant to tetracycline due to their ability to readily acquire and share *tet* genes via mobile genetic elements, which bypass tetracycline's mechanism of action.

5. Conclusion

The present study highlighted the importance of raw meat and chicken in harboring antibiotic-resistant *Enterococci* that cross-contaminate the meat during unhygienic slaughtering and dressing operations. Our study showed a high level of phenotypic resistance among *Enterococcus* species isolated from eggshell surfaces, chicken, chevon, and mutton. The current study emphasizes the need for targeted antibiotic-resistance surveillance of *Enterococci* in food animals.

6. Acknowledgment

The authors are thankful to the Indian Council of Medical Research, New Delhi, for financial support (Grant No. Zon50/2020/ECIDI), and the laboratory facilities developed through ICMR funding were used in the research.

7. Conflict of interest

The authors declare no conflict of interest

Table 1: Oligonucleotide sequences used for the detection of *Enterococcus* species

Sr. No.	Gene	Primer sequence (5' to 3')	Product size (bp)	Reference
1	16S rRNA	F-GGATTAGATACCCTGGTAGTCC R-TCGTTGCGGGACTTAACCCAAC	320	Kariyama <i>et al.</i> (2000) ^[10]
2	<i>E. faecalis</i> (<i>ddl</i>)	F-ATCAAGTACAGTTAGTCTTTATTAG R-ACGATTCAAAGCTAACTGAATCAGT	941	
3	<i>E. faecium</i> (<i>ddl</i>)	F-TTGAGGCAGACCAGATTGACG R-TATGACAGCGACTCCGATTCC	658	
4	<i>Hyl</i>	F-ACAGAAGAGCTGCAGGAAATG R-GACTGACGTCCAAGTTCCAA	276	Vankerckhoven <i>et al.</i> (2004) ^[19]

Table 2: Antibiotic susceptibility of *Enterococcus* species from chicken meat (n=51)

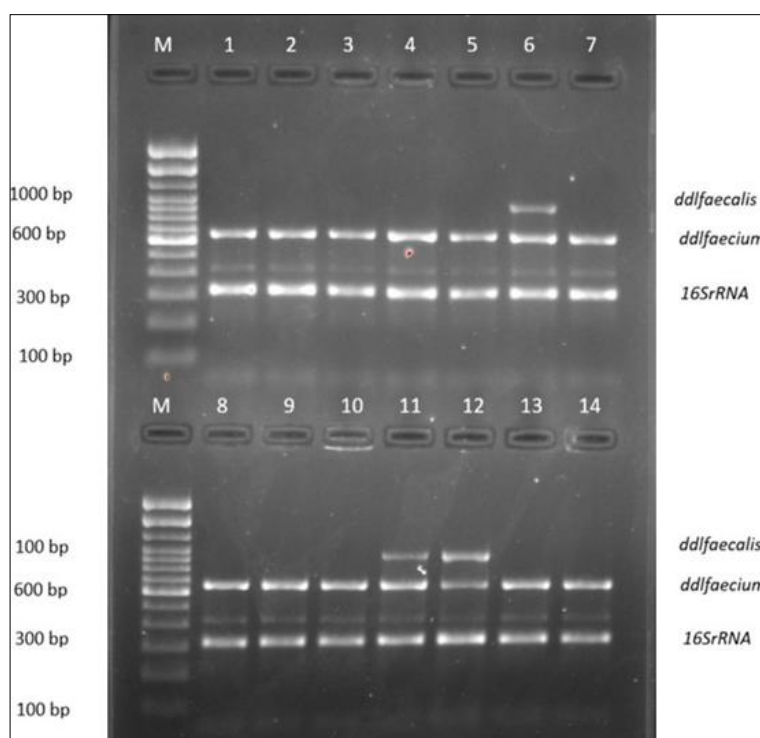
Antibiotic	R (%)	I (%)	S (%)	χ^2 (R/I/S)	p-value	95% CI for R	95% CI for I	95% CI for S
Amoxicillin clavulanate	0.0%	0.0%	100.0%	102	7.0955e-23	0.0–7.0%	0.0–7.0%	93.0–100.0%
Ampicillin	31.4%	0.0%	68.6%	36.118	1.4360e-08	20.3–45.0%	0.0–7.0%	55.0–79.7%
Chloramphenicol	23.5%	39.2%	37.3%	2.235	3.2705e-01	14.0–36.8%	27.0–52.9%	25.3–51.0%
Ciprofloxacin	19.6%	31.4%	49.0%	6.706	3.4981e-02	11.0–32.5%	20.3–45.0%	35.9–62.3%
Clindamycin	76.5%	3.9%	19.6%	44.588	2.0787e-10	63.2–86.0%	1.1–13.2%	11.0–32.5%
Doxycycline	49.0%	25.5%	25.5%	5.647	5.9396e-02	35.9–62.3%	15.5–38.9%	15.5–38.9%
Erythromycin	70.6%	27.5%	2.0%	36.824	1.0090e-08	57.0–81.3%	17.1–40.9%	0.3–10.3%
Gentamicin	13.7%	0.0%	86.3%	65.765	5.2405e-15	6.8–25.7%	0.0–7.0%	74.3–93.2%
Rifampicin	64.7%	25.5%	9.8%	24.471	4.8560e-06	51.0–76.4%	15.5–38.9%	4.3–21.0%
Tetracycline	100.0%	0.0%	0.0%	102	7.0955e-23	93.0–100.0%	0.0–7.0%	0.0–7.0%

Table 3: Antibiotic susceptibility of *Enterococcus* species from eggshell surface (n=48)

Antibiotic	R (%)	I (%)	S (%)	χ^2 (R/I/S)	p-value	95% CI for R	95% CI for I	95% CI for S
Amoxicillin clavulanate	6.2%	18.8%	75.0%	38.625	4.0991e-09	2.1–16.8%	10.2–31.9%	61.2–85.1%
Ampicillin	14.6%	2.1%	83.3%	55.125	1.0709e-12	7.2–27.2%	0.4–10.9%	70.4–91.3%
Chloramphenicol	12.5%	16.7%	70.8%	30.5	2.3824e-07	5.9–24.7%	8.7–29.6%	56.8–81.8%
Ciprofloxacin	2.1%	27.1%	70.8%	34.875	2.6729e-08	0.4–10.9%	16.6–41.0%	56.8–81.8%
Clindamycin	93.8%	6.2%	0.0%	79.125	6.5800e-18	83.2–97.9%	2.1–16.8%	0.0–7.4%
Doxycycline	12.5%	52.1%	35.4%	11.375	3.3881e-03	5.9–24.7%	38.3–65.5%	23.4–49.6%
Erythromycin	45.8%	43.8%	10.4%	11.375	3.3881e-03	32.6–59.7%	30.7–57.7%	4.5–22.2%
Gentamicin	0.0%	0.0%	100.0%	96	1.4252e-21	0.0–7.4%	0.0–7.4%	92.6–100.0%
Rifampicin	64.6%	29.2%	6.2%	24.875	3.9670e-06	50.4–76.6%	18.2–43.2%	2.1–16.8%
Tetracycline	87.5%	12.5%	0.0%	64.5	9.8629e-15	75.3–94.1%	5.9–24.7%	0.0–7.4%

Table 4: Antibiotic susceptibility of *Enterococcus* species from chevon (n=67)

Antibiotic	R (%)	I (%)	S (%)	χ^2 (R/I/S)	p-value	95% CI for R	95% CI for I	95% CI for S
Ampicillin	94.0%	0.0%	6.0%	111.433	6.3484e-25	85.6–97.7%	0.0–5.4%	2.3–14.4%
Amoxicillin Clavulanic acid	0.0%	22.4%	62.7%	47.684	4.4208e-11	0.0–5.4%	14.1–33.7%	50.7–73.3%
Chloramphenicol	13.4%	40.3%	46.3%	12.299	2.1351e-03	7.2–23.6%	29.4–52.3%	34.9–58.1%
Clindamycin	41.8%	32.8%	25.4%	2.716	2.5712e-01	30.7–53.7%	22.8–44.7%	16.5–36.9%
Ciprofloxacin	22.4%	35.8%	41.8%	3.97	1.3737e-01	14.1–33.7%	25.4–47.8%	30.7–53.7%
Doxycycline	29.9%	47.8%	22.4%	6.836	3.2781e-02	20.2–41.7%	36.3–59.5%	14.1–33.7%
Erythromycin	68.7%	31.3%	0.0%	47.493	4.8655e-11	56.8–78.5%	21.5–43.2%	0.0–5.4%
Gentamicin	0.0%	14.9%	85.1%	82.955	9.6940e-19	0.0–5.4%	8.3–25.3%	74.7–91.7%
Rifampicin	100.0%	0.0%	0.0%	134	7.9849e-30	94.6–100.0%	0.0–5.4%	0.0–5.4%
Tetracycline	100.0%	0.0%	0.0%	134	7.9849e-30	94.6–100.0%	0.0–5.4%	0.0–5.4%

**Fig 1:** Multiplex PCR for the detection of *ddlfaecalis*, *ddlfaecium*, and *16SrRNA* genes in *Enterococcus* species M- 100 bp, DNA ladder; Lanes 1-14 (*16SrRNA* gene 320 bp; *ddl E. faecium* gene 658 bp) Lanes 6,11, and 12 (*ddl E. faecalis* gene 941 bp)

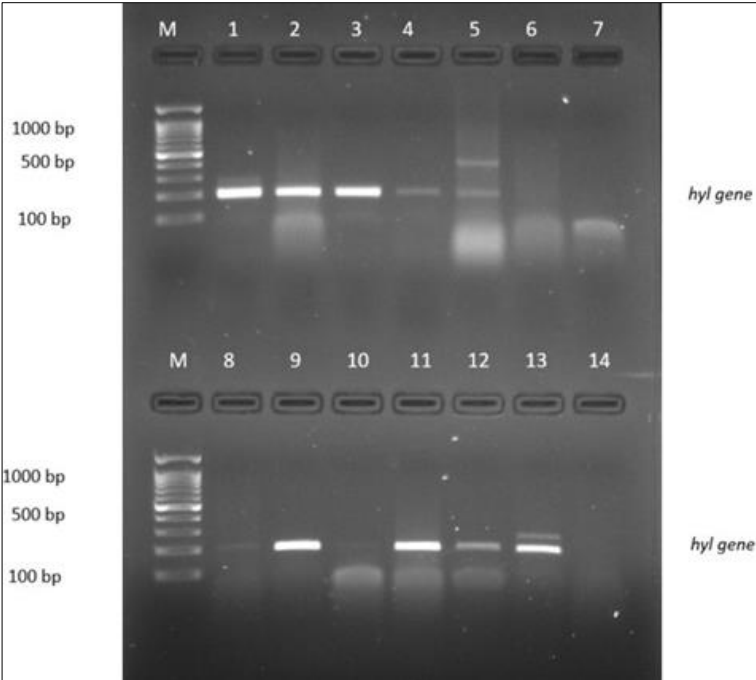


Fig 2: Detection of *hyl* gene in *Enterococcus* species, M- 100 bp DNA ladder; Lanes 1,2,3,4,5, and 9,11,12,13 (*hyl* gene 276 bp)

Table 5: Antibiotic susceptibility of *Enterococcus* species from mutton (n=83)

Antibiotic	R (%)	I (%)	S (%)	χ^2 (R/I/S)	p-value	95% CI for R	95% CI for I	95% CI for S
Ampicillin	28.9%	0.0%	71.1%	63.639	1.5173e-14	20.3–39.4%	0.0–4.4%	60.6–79.7%
Amoxicillin Clavulanic acid	0.0%	27.7%	72.3%	66.241	4.1301e-15	0.0–4.4%	19.2–38.2%	61.8–80.8%
Chloramphenicol	6.0%	42.2%	51.8%	29.012	5.0132e-07	2.6–13.3%	32.1–52.9%	41.2–62.2%
Clindamycin	50.6%	31.3%	18.1%	13.325	1.2778e-03	40.1–61.1%	22.4–41.9%	11.3–27.7%
Ciprofloxacin	12.0%	28.9%	59.0%	28.217	7.4608e-07	6.7–20.8%	20.3–39.4%	48.3–69.0%
Doxycycline	7.2%	38.6%	54.2%	28.506	6.4565e-07	3.4–14.9%	28.8–49.3%	43.6–64.5%
Erythromycin	54.2%	8.4%	37.3%	26.699	1.5938e-06	43.6–64.5%	4.1–16.4%	27.7–48.1%
Gentamicin	6.0%	12.0%	81.9%	88.651	5.6204e-20	2.6–13.3%	6.7–20.8%	72.3–88.7%
Rifampicin	83.1%	16.9%	0.0%	96.169	1.3099e-21	73.7–89.7%	10.3–26.3%	0.0–4.4%
Tetracycline	100.0%	0.0%	0.0%	166	8.9858e-37	95.6–100.0%	0.0–4.4%	0.0–4.4%

R-resistant, I- intermediate, S-sensitive, CI- confidence interval

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