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Prevalence and antibiogram study of *Enterococcus species* isolated from poultry from arid regions of Rajasthan

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Abstract
Enterococci are frequent inhabitants of GIT of poultry but can also lead to systemic infections. The purpose of this study was to isolate *Enterococcus* species (*E. faecalis* and *E. faecium)* from healthy poultry cloacal swabs and to conduct genotypic characterisation and antibiogram study. In the present study, out of 50 cloacal swab samples a total of 44 *Enterococcus* species were identified by *tuf* gene and *sod A* gene based PCR. Overall prevalence recorded was 88% and of the 44 *Enterococcus* species, 27 (54%) were identified as *E. faecium*, and 17 (34%) as *E. faecalis.* In antibiogram study, out of the 14 antibiotics used, three namely, chloramphenicol, co-trimoxazole, and trimethoprim were 100% effective against all the isolates, followed by gentamicin (97.7%) and ampicillin (95.5%). The highest resistance was shown towards tetracycline (95.5%) followed by doxycyclines (70.5%) and erythromycin (22.7%). This study highlights the diversity of *Enterococcus* in the food chain and the need for routine antibiotic resistance surveillance in food animals such as poultry.

Keywords: Antibiogram, *Enterococcus faecalis*, *Enterococcus faecium,* poultry, prevalance

Introduction
Due to the rising demand for poultry products, particularly in urban areas due to their high food value, poultry farming has gained great importance. The gastrointestinal tracts of poultry are highly colonized with complex microbial communities (bacteria, fungus, archaea, protozoa, and virus), with bacteria predominating in these groups (Wei *et al*., 2013) [36]. The antimicrobial-resistant microbes which are present in animal faecal material transfer genes to human beings by contaminated feed and water supplies. In poultry enterococci, mainly *E. faecalis* and *E. faecium* are normally present as commensals but pathogenic strains associated with arthritis, spondylitis, femoral head necrosis, osteomyelitis, lameness and paralysis of broilers have also been reported (Stalker *et al.,* 2010, Velkers *et al.,* 2011) [30, 35]. Compared to other intestinal microbes like coliforms, *Enterococcus* are resilient and can survive for longer duration in soil and water, thus *Enterococcus* have more possibility to re-enter the food web (Boyce, 1997) [5].
The use of antibiotics has increased at sub-therapeutic levels in animal production as feed additives for growth promotion (Ronquillo and Hernandez, 2017) [27]. Sub-therapeutic antibiotic levels induce a selective pressure to antimicrobial resistance (AMR) in chicken coliform bacteria (Angulo *et al*., 2000) [1]. This high antibiotic usage in poultry may not only compromise veterinary therapy but is also of public health concern (Van den Bogaard, 2000) [34]. Hence, this study was designed for identification of *E. faecalis and E. faecium* isolates on phenotypic and genotypic level and determine the antimicrobial susceptibility against various antimicrobial agents.

Materials and Methods
Collection of samples: A total of 50 faecal samples were taken either directly from the cloaca of poultry using sterile swabs or fresh faecal matter was collected in sterile test tubes from Poultry farm, CVAS, RAJUVAS (Bikaner) and local poultry farm at Ajmer.

The samples were immediately transferred on ice in sealed containers and processed within 3-4 hours.

Isolation of *Enterococcus species*

The isolation and identification of the organism was done on the basis of cultural, morphological and biochemical characteristics as per the method described by Cowan and Steel, 1974 [6]; Quinn *et al.*, 1994 [26]. Selective plating was done after 16 hours of enrichment of faecal samples in buffered peptone water and cultures were swabbed and streaked onto *Enterococcus* selective agar (Pfizer Enterococcal Hi Veg Agar) and incubated under microaerophilic conditions at 37 °C for 48 h. Pure colonies were obtained by successive sub-culturing onto *Enterococcus* agar plates having the same incubation conditions. VITEK 2 GP IDENTIFICATION SYSTEM was used for biochemical profiling of isolates.

Molecular characterisation of *Enterococcus* species

DNA isolation was carried out as per the method of Nachimuttu *et al.* (2001) [23] from *Enterococcus* isolates with some modifications. The genotypic confirmation was carried out using *tuf* gene primers as per Ke *et al*. (1999) [18] followed by species specific identification using primers for sod AFL and sod AFM genes (Table 1) as described by Jackson *et al.* (2004) [14].

The quantity and concentration of the PCR components was same for the three genes except primers. The master mix was prepared by mixing GENETAQ Green Master Mix (2X) 12.5mL, primers (25 pM/µl) 0.5 µl each, DNA template 3.0 µl and nuclease free water to make 25.0 µl. The PCR cycle included pre denaturation at 95 °C for 5 min, 35 cycles of three steps (Denaturation at 94 °C for 40s, annealing at 55 °C for 60s and extension at 72 °C for 75s) and final extension at 72 °C for 7 min.

Table 1: List of primer pairs used for amplification of target genes in present study.

Gene	Sequence (5`-3`)	Anneal. Temp. (°C)	Product size (bp)	Reference
Tuf	F-TACTGACAAACCATTCATGATG R-AACTTCGTCACCAACGCGAAC	55	112	Ke *et al.* (1999) [18]
sodA FL	F-ACTTATGTGACTAACTTAACC R-TAATGGTGAATCTTGGTTTGG	55	360	Jackson *et al.* (2004) [14]
sodA FM	F-GAAAAAACAATAGAAGAATTAT R-TGCTTTTTGAATTCTTCTTTA	55	215	

Antimicrobial susceptibility testing

The Kirby Bauer disk diffusion technique (Bauer *et al.,* 1966) [4] was used to evaluate the antibiogram of the bacterial isolates to different antibiotics used in the study. The isolates were inoculated in sterile 5 ml brain-heart infusion broth, incubated for 18 h at 37 °C and then the opacity was adjusted to 0.5 McFarland opacity standards with Normal saline solution (Quinn *et al*., 1994) [26]. The suspension was well swabbed over the surface of Mueller Hinton agar plates with the use of sterilized swab and left to dry at room temperature for 20 minutes. Paper disks with the specific concentration of antibiotics were placed on the surface of agar plates. Plates were then incubated overnight at 37 °C. Various categories of antibiotics were used in order to study the antibiogram for *Enterococcus* isolates as described in table 2:

Table 2: List of antibiotics used for antibiogram study against *Enterococcus* isolates obtained from poultry

S. No	Antibiotic/Symbol (Interpretation zone in mm- R/I/S)	Disc content (mcg)
	Ampicillin/AMP^{2}(8/9/10)	10
	Bacitracin/B^{10} (8/9-12/13)	10
	Chloramphenicol/C^{30}(12/13)	30
	Ciprofloxacin/CIP^{5} (15/16-20/21)	30
	Co-trimoxazole/COT^{25} (10/11-15/16)	30
	Doxycycline/DO^{30}(12/13-15/12)	30
	Erythromycin/E^{15} (13/14-22/23)	05
	Gentamicin/ HLG^{120} (6/7-9/10)	120
	Levofloxacin/ LE^{5} (13/14-16/17)	05
	Penicillin G/ P^{10} (14/-/15)	10 Units
	Rifampicin / RIF^{5} (16/17-19/20)	05
	Tetracycline / TE^{30} (14/15-18/19)	30
	Trimethoprim / TR^{5}(10/11-15/16)	05
	Vancomycin /VA^{5} (14/15-16/17)	05

Reading of sensitivity test results

The zone of inhibition of growth was measured using a transparent ruler and recorded to the nearest millimeters. According to a standard chart provided by the disc manufacturer, the inhibitory zone readings were used to determine whether the isolates were resistant, intermediate, or sensitive to antibiotics.

Results and discussion

Prevalence and occurrence of *Enterococcus* species in poultry

In the present study, a total of 50 faecal samples were taken either directly from the cloacae of poultry using sterile swabs or fresh faecal matter was collected in sterile test tubes. A total of 30 samples were collected from poultry belonging to poultry farm at CVAS, RAJUVAS, Bikaner, Rajasthan and 20 from local poultry farm at Ajmer, Rajasthan. Out of the 50 samples collected, 48 enterococci were presumptively identified by conventional methods and *tuf* gene-based genus-specific PCR (Fig: 1) with an overall recovery rate of 96%. Out of 48 enterococci isolated, 17 (34%) *Enterococcus faecalis* and 27 (54%) *E. faecium* isolates were confirmed using *sod*A gene-based species-specific PCR (Fig: 2,3). Hence a total of 44 *Enterococcus* species including 17 *E. faecalis* and 27 *E. faecium* isolates were identified out of 50 samples with a recovery rate of 88%. In the present study, an overall high prevalence of *E. faecium* in poultry cloacal samples was observed from both the places of study. The predominance of *E. faecium* over *E. faecalis* isolates from poultry cloacal swabs in the present study is similar to the findings of Kim *et al.* (2019) [19], Sanlibaba *et al.* (2018) [29], Unal *et al.* (2017) [33]. Contrary to present findings *some scientists have reported* a higher prevalence of *E. faecalis* than *E. faecium* in poultry *viz.* Molechan *et al.* (2019) [22], Aslantas (2019) [3], Jangir *et al.* (2015a) [15]. These discrepancies among reported prevalence rates of enterococci could be attributed to the differences in the geographical location, farm environment and managemental practices (Liu *et al*., 2013) [20] as well as the method of sampling (Nowakiewicz *et al*., 2017; Obeng *et al*., 2013) [24, 25].

Cultural and biochemical properties

A total of 48 strains were presumptively identified as enterococci. Colonies obtained after 24-48 h of partial anaerobic incubation were pinpoint grayish-black centered with a halo around. All of the 48 enterococcal isolates in the

present study were Gram (+) (Fig 2), catalase (−), and esculin hydrolysis (+) (Fig. 3) and showed optimum developmental characteristics at 6.5% NaCl and at 10–45 °C. Similar findings were also observed by Isenberg *et al.* (1970) [13], Jangir *et al.* (2015a) [15] and Sanlibaba *et al*. (2018) [29]. Out of 48 presumptively isolated isolates 44 were identified by VITEK2 GP IDENTIFICATION SYSTEM with excellent identification with 92-98% probability. A total of 44 enterococci confirmed using *sod*A gene-based PCR included 17 *E. faecalis* and 27 *E. faecium* isolates.

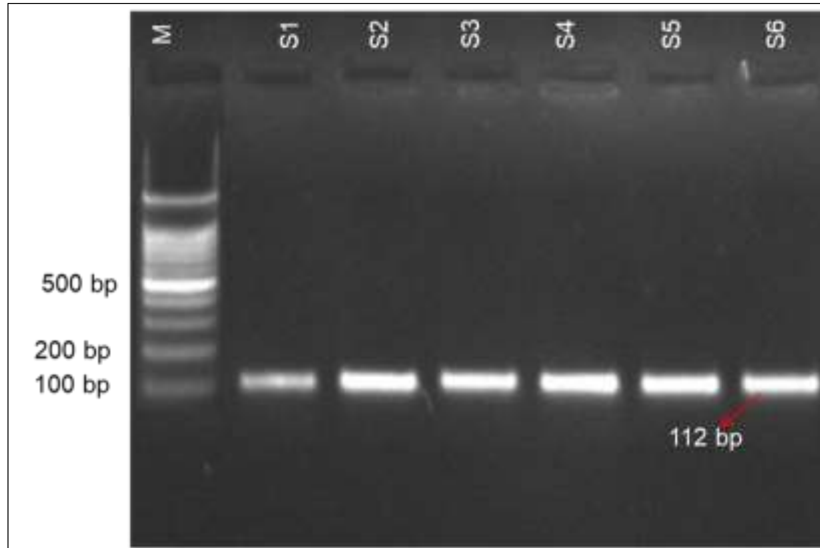

Fig 1: Gel image showing 112 bp PCR amplicons of *tuf* gene for *Enterococcus* genus identification; M: molecular marker (100 bp)

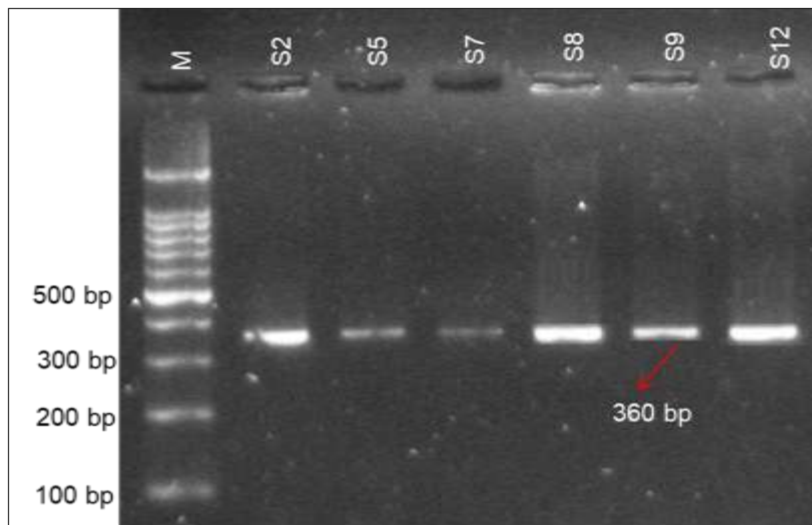

Fig 2: Gel image showing 360 bp PCR amplicons of *sodAfs* gene of *Enterococcus faecalis*; M: molecular marker (100 bp)

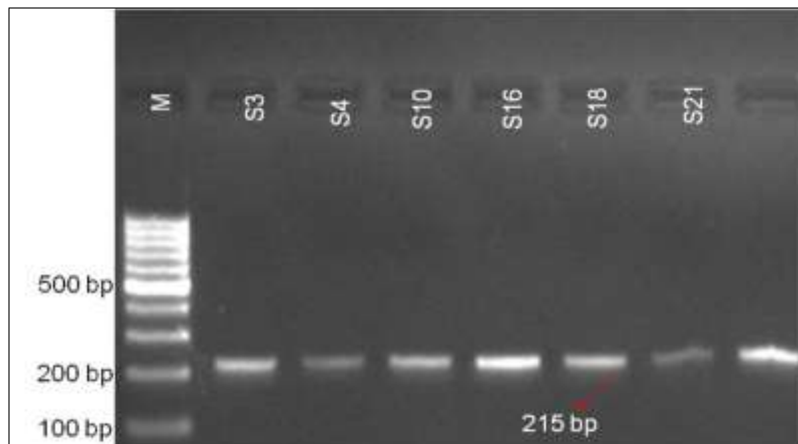

Fig 3: Gel image showing 215 bp PCR amplicons of sod Afm gene of *Enterococcus faecium*; M: molecular marker (100 bp)

Antibiogram study: In the present investigation 14 antibiotics belonging to ten different classes were used to carry out the antibiogram study (Fig. 4). A summary of the resistance among the *Enterococcus* isolates is reported in Table 3.

Table 3: Antibiotic sensitivity profiling of *Enterococcus* isolates from poultry cloacal swabs

S. No.	Antibiotic Disc	Antibiogram pattern (%)								
		Enterococcus faecalis			*Enterococcus Faecium*			Total		
		(R)	(I)	(S)	(R)	(I)	(S)	(R)	(I)	(S)
1.	Chloramphenicol	-	-	17 (100)	-	-	27 (100)	-	-	44 (100)
2.	Co-trimoxazole	-	-	17 (100)	-	-	27 (100)	-	-	44 (100)
3.	Trimethoprim	-	-	17 (100)	-	-	27 (100)	-	-	44 (100)
4.	Gentamicin	-	-	17 (100)	-	01 (3.7)	26 (96.3)	-	01 (2.3)	43 (97.7)
5.	Ampicillin	-	-	17 (100)	2 (7.4)		25 (92.6)	02 (4.5)	-	42 (95.4)
6.	Levofloxacin	-	02 (11.8)	15 (88.2)	-	04 (14.8)	23 (85.2)	-	06 (13.6)	38 (86.4)
7.	Ciprofloxacin	-	03 (17.6)	14 (82.3)	-	04 (14.8)	23 (85.2)	-	07 (15.9)	37 (84.1)
8.	Rifampicin	-	02 (11.8)	15 (88.2)	-	05 (18.5)	22 (81.5)	-	07 (15.9)	37 (84.1)
9.	Vancomycin	-	06 (35.3)	11 (64.7)	-	07 (25.9)	20 (74.1)	-	13 (29.5)	31 (70.4)
10.	Bacitracin	-	06 (35.3)	11 (64.7)	01 (3.7)	07 (25.9)	19 (70.4)	01 (2.3)	13 (29.5)	30 (68.2)
11.	Penicillin-G	06 (35.3)	-	11 (64.7)	12 (44.4)	-	15 (55.5)	18 (40.9)	-	26 (59.1)
12.	Erythromycin	03 (17.6)	05 (29.4)	09 (52.9)	07 (25.9)	05 (18.5)	15 (55.5)	10 (22.7)	10 (22.7)	24 (54.5)
13.	Doxycycline	10 (58.8)	05 (29.4)	02 (11.8)	21 (77.8)	04 (14.8)	02 (7.4)	31 (70.4)	09 (20.4)	04 (9.1)
14.	Tetracycline	15 (88.2)	02 (11.8)		27 (100)	-	-	42 (95.4)	02 (4.5)	-
Abbreviations R- resistant; I- intermediate; S- sensitive										

Of the 14 antibiotics used, three namely, chloramphenicol, co-trimoxazole, and trimethoprim were detected to be 100% effective against all the isolates, followed by gentamicin and ampicillin (97.7%) and (95.5%) isolates, respectively whereas other antibiotics showed lower efficacies. The highest resistance was shown towards tetracycline (95.5%) followed by doxycyclines (70.5%). Moreover, a certain percentage of isolates exhibited intermediate sensitivity, particularly to ciprofloxacin (15.9%), rifampin (15.9%), levofloxacin (13.6%), and gentamicin (2.3%).

Fig 4: Antibiotic sensitivity test on Mueller Hinton agar

In the present study, most of the *Enterococcus* isolates regardless of the species were resistant to the tetracycline group of antibiotics which are frequently used for treating infections. Similar to the present study, high tetracycline resistance was reported by other workers from different parts of the world *viz*. Aslantas, (2019) [3], Molecham *et al.* (2019) [22]. Nowakiewicz *et al.* (2017) [24], Unal *et al.* (2017) [33], Dilik *et al*. (2010) [8]. High levels of tetracycline resistance may be attributed to the extensive use of tetracycline analogs for food animals, which can create a selective environment for subsequent resistance (Eagar *et al.,* 2012) [9]. Hence therapeutic and prophylactic use of tetracycline in broilers feeds is a common cause of resistance (Michalova *et al.,* 2004) [21].

Resistance to penicillin-G was observed in 18 (40.90%) of the total *Enterococcus* isolates which comprised of 12 (66.7%) *E. faecium* isolates and six (33.3%) *E. faecalis* isolates. A similar resistance pattern was observed by Jangir *et al.* (2015b) [16] from the same area of study. Hayes *et al.* (2004) [12], Ruzauskas *et al.* (2009) [28] and Furtula *et al.* (2013) [10] also reported penicillin resistance on poultry originated samples. While no penicillin-resistant strains was observed by Tejedor-Junco *et al.* (2005) [32] which is in contrast to present findings. As compared to the present study, high resistance to erythromycin has been reported ranging from 38.14% by Sanlibaba *et al.* (2018) [29] to 100% by Guerrero-ramos *et al.* (2016) [11]. A combination of gentamicin with cell wall active antibiotics (e.g., beta-lactams, vancomycin) has been used for the treatment of enterococcal infections, e.g., endocarditis, bacteremia, and meningitis. However, this combination is not effective in the treatment of infections caused by enterococci with high-level gentamicin resistance (HLGR) and so HLGR is clinically important (De Jong *et al.* 2018) [7]. In the present study, since all the isolates included were of non- clinical origin, no isolate was observed to show resistance to high-level gentamicin and 97.7% isolates were sensitive that is similar to previous studies conducted by Guerro-ramos *et al.* (2016) [11] and Molecham *et al.* (2019) [22].

Conclusion

A high prevalence of *Enterococcus* spp. was observed in cloacae of healthy poultry. The *tuf* gene and *sodA* gene based PCR was effective in identifying the isolates at genus and species level. A high resistance to tetracyclines was also observed. Potential transfer of these genes from poultry to humans by pathogenic enterococci occurs through the food chain, thus, underscores the need for routine antibiotic resistance surveillance in food animals.

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