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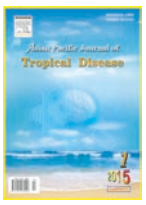

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Infection dynamics of vancomycin and inducible clindamycin resistant *Enterococcus faecalis* in an Indian teaching hospital

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ABSTRACT

Objective: To do surveillance for vancomycin and inducible clindamycin resistance of *Enterococcus faecalis* (*E. faecalis*), a Gram-positive bacterium in a teaching hospital.
Methods: *E. faecalis* strains isolated from clinical samples were screened for vancomycin and inducible clindamycin resistance, *i.e.*, D-test positivity, using vancomycin screen agar and blood agar plates, respectively. For the D-test screening, erythromycin resistant (Er-r) and clindamycin sensitive (Cd-s) strain were used.
Results: Of 265 isolated *E. faecalis* strains, 159 (60%) were vancomycin resistant *Enterococcus* (VRE) and 106 were vancomycin sensitive *Enterococcus* (VSE). Of 265 strains, 42 were constitutively resistant to clindamycin and erythromycin and of 148 Er-r and Cd-s strains, 87 (32.83%) had D-test positivity, while the rest 61 strains were D-test negatives. D-test results examined with 6 hospital factors as bivalents, only 2 factors, the VSE/VRE and the presence/absence of prior antibiotic use > 90 days bivalent were statistically significant. A VRE strain with D-test positivity would be picked up 0.5702 times more frequently than a strain with VSE and D-test positivity. Also, patients with prior antibiotic use > 90 days had 3.7375 times more chance of picking up D-test positive strains than patients without any prior antibiotic use. Resistance pattern of *E. faecalis* strains to individual 14 antibiotics were recorded; the maximum values of resistance were against ampicillin 10 μg/disc and linezolid 30 μg/disc. Student's *t*-test for hospital acquired and community acquired data revealed that drug resistant strains were equally prevalent in both sources.
Conclusions: Prevalence of 60% VRE in both hospital and adjoining community creates consternation. In total 87 (32.83%) strains had D-test positivity; patients who had used antibiotics within the last 90 days have got an ample chance of picking of D-test positive *E. faecalis*. D-test protocol should be followed with clinical samples in hospitals for Gram-positive bacteria.

1. Introduction

Gram-positive (GP) enterococci along with *Lactobacillus* sp. have been in use in food industry during the fermentation of dairy products. The cheese industry has been using *Enterococcus faecalis* (*E. faecalis*)[1], whose prodigal strains have been identified as causatives of food spoilage. Thus, the dualistic role of enterococci had been recorded in both adjunct culturing process and food spoilage[2]. Furthermore, *E. faecalis* strains are the frequently confronted pathogenic bacterium, in suppurative infections. For example in the local hospital setting, along with enterococci, two other genera staphylococci and streptococci have developed multidrug resistant (MDR) phenotypes equally rather in a more disturbing proportion compared to Gram-negative bacteria[3,4]. Antibiotics belonging to macrolides, lincosamides and

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streptogramin B (MLSB) groups are preferably used against any GP infection. A strain with erythromycin (macrolide) resistance along with clindamycin sensitivity [erythromycin resistant (Er-r) and clindamycin sensitive (Cd-s)], when plated with clindamycin discs on a lawn of the bacterium, a zone of inhibition around clindamycin disc is expected. But, clindamycin resistance induced by the *Er-r* gene towards the Er-disc results in a partial inhibition of inhibition zone around clindamycin disc forming the characteristic D-flattening, consequently known as the characteristic D-test (Figure 1) or D-test positivity. Thus, clindamycin would be ineffective in controlling Cd-s strains in the presence of the Er-r character, due to the presence of *MLSB* gene. The inducible clindamycin resistance of the most common GP pathogen, *Staphylococcus aureus* (*S. aureus*) had been independently reported from several geographic zones[5-7]. *Enterococcus* sp. has been seen to have susceptibility to erythromycin partially, rendering it to continue as the control agent of an unidentified GP infection empirically by clindamycin. Thus, *Enterococcus* sp. had two types of strains, erythromycin sensitive (Er-s) and Er-r. D-test positive strains of *E. faecalis* can contribute to life-threatening situations in nosocomial sector by producing a plasmid encoded haemolysis with cytolysin, which had been verified in an animal system[8].

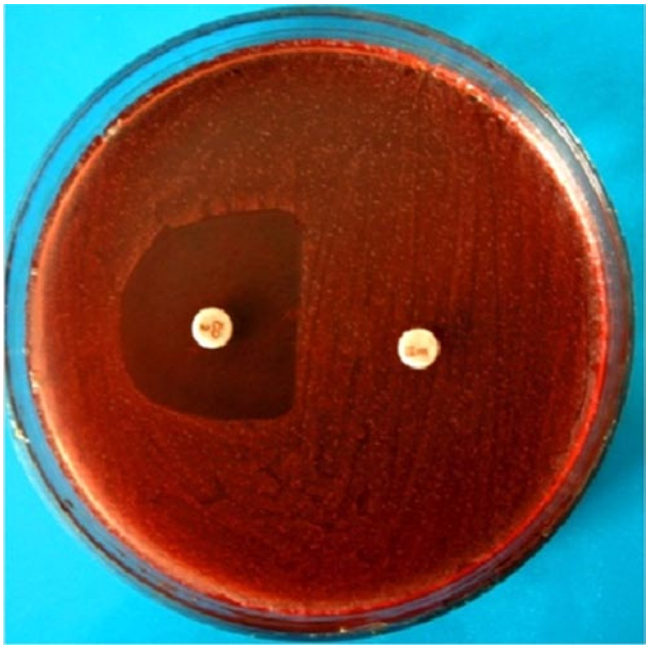

Figure 1. D-shape flattening of clindamycin sensitive zone of *E. faecalis* in the lawn of Er-r and Cd-s strain, induced by erythromycin disc.

Recently with increasing resistance to aminoglycosides and β-lactams, treatments with vancomycin were introduced for the control of infections by enterococci, often empirically. However, several strains of enterococci live in patients non-symptomatically, as this bacterium is a commensal, but its drug-resistant strains remain pathogenic and serve as sources for nosocomial infections. Indeed, the first report on vancomycin resistant enterococci (VRE), *i.e.*, *E. faecalis* was from northern India, which had 26% aminoglycoside resistant strains, while 66% resistance to penicillin, 88% resistance to ciprofloxacin, 85% resistance to erythromycin along with 1% resistant to vancomycin[9]. The inherent difficulty with VRE is the failure to access the harmless colonizing strains from an infection. Therefore, detection of vancomycin sensitivity has been a method to differentiate between harmless colonization and harmful infection[9].

For the control of pathogenic *E. faecalis,* clindamycin (a lincosamide) is used empirically, before the result of culture of a clinical sample is obtained for a patient with primary infections such as, urinary tract infection UTI, injuries, surgical sites and suppurative infections, which when uncontrolled initially, lead to meningitis, endocarditis and a few more life threatening diseases through pyelonephritis and blood stream infections. The general surveillance of this hospital for *S. aureus* was done previously[4]. In continuation to the inducible clindamycin resistance work with *S. aureus*[7], this study was unwittingly pursued to probe D-test positive *E. faecalis* strains, particularly in this well-attended philanthropic hospital. A failure in control of an infection by any antibiotic empirically would be a medical infraction, because there would be progress of the insinuating bacterium internally to innards during 3-4 days, till the culture report is available.

Both vancomycin resistance and D-test positivity had been considered in surveillance of *E. faecalis* with 265 isolates in a tertiary care hospital from clinical samples, since *E. faecalis* has come up to an ill-famous notoriety with the armamentarium of multidrug resistance. Moreover, a univariate analysis of the bivalent of D-test results for a few hospital factors was undertaken, which gave a conclusion that conducting D-test for clinical samples would be a prudent approach for hospital management, in face of inducible clindamycin resistance in GP pathogens. This study recorded both VRE and vancomycin sensitive *Enterococcus* (VSE) strains from hospital and community sectors. Herein, antibiograms of 265 isolates with 14 antibiotics had been done and other factors related to D-test positivity were considered, which gave a picture of insidious infection dynamics of *E. faecalis*. This work should help apothecary in dovetailing suitable drugs to decrease unwarranted burden in the growing cost of hospital care, morbidity and mortality.

2. Materials and methods

2.1. Isolation and identification

The study was conducted for a period of 6 months (June to November 2013) and a total of 265 strains of *E. faecalis* were isolated from different clinical samples from hospital acquired (HA) and community acquired (CA) sources. Isolated strains were identified by using the standard microbiological procedures[10]. The standard *E. faecalis* sensitive strain, number 439 from Microbial Type Culture Collections (MTCC) was used as the reference control.

Catalase negative, alpha-haemolytic (partial or green haemolysis of erythrocytes) grey, small and round colonies were subjected to bile-esculin test; ferric citrate was added after growth as a colour-indicator. The bile-esculin medium contains esculin and peptone for nutrition and bile to inhibit growth of GP bacteria, in other than streptococci or enterococci. Organisms, which split from esculin molecules and use the liberated glucose to supply energy, release esculin into the medium. The free esculin reacts with ferric citrate in the medium to form a phenolic iron complex, which turns the agar-slant from dark brown to black. An agar-slant that was more than half darkened within 48 h of incubation was bile-esculin positive, for the confirmation of *E. faecalis*; but the alternative non-darkening of the agar was taken as the negative result[10].

2.2. Detection of vancomycin resistant strains

Vancomycin screen agar plate was prepared by addition of 6 μg/mL vancomycin to brain heart infusion agar (HiMedia). Inoculum suspension was prepared for the turbidity of a 0.5 McFarland standard, from which an aliquot of 0.1 mL of this suspension was spread onto a vancomycin-screen agar plate and it was incubated for 24 h at 37 ˚C in bacteriological incubator. Any visible growth indicated the vancomycin resistance[10]. In addition, the *E. faecalis* MTCC 439 strain was used as the vancomycin-susceptible control.

2.3. D-test

Isolates of Er-r and Cd-s were tested for possible inducible clindamycin resistant (Cd-r), to probe susceptibility to clindamycin 2 μg/disc and erythromycin 15 μg/disc levels, along with the reference strain, according to Clinical And Laboratory Standards Institute criteria. Erythromycin and clindamycin discs (HiMedia) were placed (17 ± 2) mm apart (edge to edge) on a blood agar plate, incubated at 37 ˚C for 18 h and D-test positivity was identified by the D-type flattening of clindamycin zone towards the erythromycin disc (Figure 1)[7].

2.4. Antibiotic susceptibility test

All isolated strains including the standard MTCC strain of *E. faecalis* were subjected to antibiotic sensitivity tests by the Kirby-Bauer's/disc diffusion method, using a 4 mm thick Mueller-Hinton agar (HiMedia) medium. An aliquot of 0.1 mL of 0.5 McFarland equivalents, approximately from an exponentially growing culture was spread on agar for the development of lawn at 37 ˚C in a BOD incubator (Remi CIM-12S). Further, on the lawn-agar of each plate, 8 high potency antibiotic discs (HiMedia, Mumbai) of 14 prescribed antibiotics were placed at equal distances from one another. Plates were incubated for 18 h at 37 ˚C and were examined for size of zones of inhibition around each disc, following the standard antibiotic susceptibility test chart of Clinical Laboratory Standard Institute guidelines[5,11].

3. Results

A total of 265 *E. faecalis* strains were isolated from clinical samples, pus, swabs, urine, body fluids and blood, in the cited order of prevalence, from both from HA and CA sources (Table 1). Of 265 (100%), 170 (64.15%) isolates were isolated from HA and 95 (35.84%) from CA samples. Of 170 HA isolates, 102 (38.49%) strains were VRE and 68 (25.66%) were VSE, whereas of the total 95 CA strains, 57 (21.15%) were VRE and 38 (14.33%) were VSE (Table 1 and Figure 2).

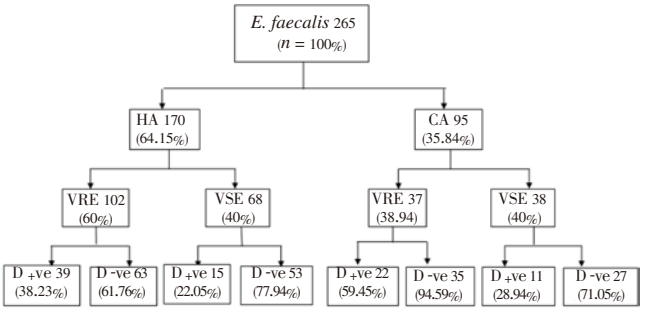

Figure 2. Prevalence of D-test positive and negative colonies, along with vancomycin sensitive/resistant isolates of *E. faecalis*.

Table 1
Occurrence of inducible clindamycin resistant strains using Er-r and Cd-s *E. faecalis* strains among vancomycin sensitive and vancomycin resistant strains, isolated from clinical samples.

Clinical samples	HA		CA	
	VSE	VRE	VSE	VRE
Pus	10 (2)	29 (10)	9 (3)	14 (8)
Wound swabs	17 (3)	14 (8)	11 (5)	3 (3)
Skin swabs	8 (5)	13 (8)	12 (3)	15 (5)
Nasal swabs	5 (-)	4 (1)	- (-)	- (-)
Urine	18 (3)	11 (2)	3 (-)	12 (3)
Body fluids	1 (-)	7 (4)	- (-)	03 (2)
Blood	9 (2)	24 (6)	3 (-)	10 (1)
Total	68 (15)	102 (39)	38 (11)	57 (22)

Numbers in parentheses represent D-test positives (inducible clindamycin resistant) strains that were total being (15 + 39 + 11 + 22, 87), out of the grand total of (68 + 102 + 38 + 57, 265) isolates.

Of types of combination of resistant/sensitive strains, constitutive resistant (Er-r, Cd-r), constitutive sensitive (Er-s, Cd-s) and D-test positive or negative (Er-r, Cd-s) were tested for vancomycin sensitivity as well as, D-test positivity. It was found that constitutive resistant strains were totally vancomycin resistant and they were in total 42 strains, with 29 from HA and 13 from CA source. The constitutive sensitive strains were higher in both HA (24) and CA (36) sources; but there were 7 and 8 VRE strains with constitutive sensitive isolates, from HA and CA sources. Both D-test negative and D-test positive strains had 148 (61 + 87) as the total strains from HA and CA samples of which, both VSE and VRE strains from HA and CA sample had only 87 positive strains. Summarily stating, 41 + 75 + 61 = 177 were

D-test negative strains (Table 2).

Table 2
Types of phenotypes among vancomycin sensitive and vancomycin resistant strains in 265 *E. faecalis* concerning to resistance to erythromycin and clindamycin.

Strains	HA		CA		Total
	VSE	VRE	VSE	VRE	
Er-r + Cd-r Constitutive resistant	0	29	0	13	42
Er-s + Cd-s Constitutive sensitive	24	7	36	8	75
Er-r + Cd-s D-test negative	14	21	15	11	61
Er-r + Cd-s D-test positive	19	27	12	29	87

Of the total 265 strains, 87 (32.83%) showed D-test positivity and 178 (67.17%) were D-test negative. D-test positivity/negativity had concern to other variables in hospital as detailed; therefore, the total 265 strains were checked for six variables as bivalents (Table 3): 1. VSE/VRE; 2. HA/CA sources; 3. males/females; 4. presence/absence of associated comorbidities (presence/absence of diabetes-related ailments any other problems such as cardiac complaints, noteworthy diseases, *etc*.); 5. presence/absence of related infections; and 6. patient history of prior antibiotic uses within > 90 days. The *P* value for the bivalence for VSE/VRE pair was 0.0445, which was statistically significant. However, the bivalent data for HA/CA sources were statistically not significant, with the *P* value = 0.0669. Similarly, the D-test negative/positive cases related to sex at *P* = 0.3026, associated comorbidities at *P* = 0.1454 and related infections at *P* = 0.1590 were not statistically significant. On the contrary, for the bivalent with prior antibiotic use > 90 days, at *P* value = 0.0001 was highly significant statistically, signifying there were statistically respectable differences in causing D-test positive with 2 bivalents only (Table 3).

Table 3
Univariate analysis of D-test positive and negative 265 strains of *E. faecalis*, influenced by 6 variables.

Variables		D-test positive	D-test negative	*P* value	Odds ratio	95 *CI* (%)
Strains	VSE	54	132	0.0445*	0.5702	0.3297-0.9863
	VRE	33	46			
Sources	HA	52	85	0.0669	1.6255	0.9667-2.7335
	CA	35	93			
Sex	Male	47	108	0.3026	0.7616	0.4537-1.2783
	Female	40	70			
Associated comorbidities	Present	30	46	0.1454	1.5103	0.867-2.6309
	Absent	57	132			
Related infections	Present	52	122	0.1590	0.6820	0.4003-1.1617
	Absent	35	56			
Prior antibiotic use > 90 days	Present	62	71	0.0001*	3.7375	2.1502-6.4964
	Absent	25	107			

*: Statistically significant; *CI*: Confidence interval.

From the odd ratio computation, it is evident that *E. faecalis* strain with VRE would be picked up 0.5702 times more frequently than an *E. faecalis* strain with VSE; in other words vancomycin resistance prevents (1-0.5702) = 0.4298 times the spread of a D-test positive strain with vancomycin sensitivity. Similarly, hospitalized patients could pick of D-test positive strains by 1.6255 times more frequently than the people in community. Males can pick up D-test positive strains by 0.7616 times more frequently than females. People with associated comorbidities have 1. 5103 times more chance to pick up D-test positive strains than those without associated comorbidities. Presence of related infections has 0.6820 times more chance than without related infection. Prior antibiotic use within 90 days has a high level of chance, *i.e.*, 3.7375 times more chance to pick of D-test positive strains (Table 3).

In parallel, resistant patterns of all *E. faecalis* strains were studied with 14 antibiotics belonging to 7 groups. It had been recorded that, the resistance pattern to individual antibiotic were high with for example, the maximum values ampicillin (10 μg) for HA and CA isolates and least value of drug sensitivity were recorded for linezolid (30 μg) (Table 4). Since, data were appeared marginally different, Student's *t*-test was conducted, which ended up with the computed *t*-value as 0.3495, while the tabulated t-value is 2.16, degree of freedom = 13, at *P* = 0.05, which prompted for accepting the null hypothesis. Thus, the prevalence of drug resistant strains of 14 antibiotics of *E. faecalis*, obtained from HA and CA sources were almost similar.

Table 4
Resistance of 265 *E. faecalis* strains from hospital and community acquired sources to 14 antibiotics of 7 groups.

Antibiotic group	Antibiotics (μg/disc)	HA isolates (%)	CA isolates (%)
Aminoglycosides	Amikacin 30	69	64
	Gentamicin 10	77	55
β-lactams	Amoxyclav 30	65	66
	Ampicillin 10	89	78
	Penicillin 10	56	76
Fluoroquinolone	Gatifloxacin 05	68	67
Glycopeptides	Teicoplanin 10	70	69
	Vancomycin 30	48	64
Lincosamide	Clindamycin 02	62	66
Macrolides	Azithromycin 15	82	64
	Erythromycin 15	80	57
Stand-alone antibiotics	Chloramphenicol 30	62	61
	Linezolid 30	37	47
	Tetracycline 30	55	64

For HA and CA percent resistance values; Student's *t*-test = 0.3495.

4. Discussion

When plated with erythromycin and clindamycin discs on a lawn of the bacterium, the Er-r and Cd-s strains are expected to have total control with a full zone of inhibition around the clindamycin disc. But, among 265 *E. faecalis* strains, 148 were Er-r and Cd-s, of which 87 strains had clindamycin resistance in the presence of erythromycin with the characteristic D-flattening towards the erythromycin disc (D-test positive). Thus, clindamycin was ineffective in controlling Cd-s strains in the presence of Er-r strains, due to presence of *MLSB* gene with 87 strains. Clearly, the rest 148-87 = 61, Er-r and Cd-s strains were D-test negative, lacking the *MLSB* gene. Further, considering six variable factors, it was recorded that the factor, prior antibiotic uses > 90 days was conducive to acquire Er-r strain with *MLSB* gene, *i.e.*, such patients were vulnerable to picking infection with a D-test positive strain. The considerably high level of resistance in *E. faecalis* strains isolated from HA and CA clinical samples clearly indicated that options for

the use of other antibiotics for control among the major 9 groups remain limited. Of 14 antibiotics used, linezolid 30 μg/disc was the most effective antibiotic in controlling VRE and Er-r *E. faecalis* strains. Thus, the infection dynamics was discernible, indicating *E. faecalis* as the silently violent pathogen, since 265 isolated strains were floridly MDR. The increasing resistance to vancomycin had surprisingly higher levels of percentage in CA samples, compared to that of HA isolates; that 60% of total isolated strains was VRE is a matter of consternation. Er-r strains from HA samples were having prevalence at 80% compared to that of CA samples at 57%. Thus, this proves our notion that longer hospital stay brings extra infections than in community.

The methylase gene, *erm(A)*, formerly termed as *erm(TR)* was identified in *Streptococcus pyogenes* (*S. pyogenes*) conferring erythromycin resistance[12]. A work from Spain recorded different species of *Enterococcus* having erythromycin resistance, with genes, *erm(A), erm(B), erm(C) erm(TR), mef(A/E)* and *msr(A)*[13]. Each group has 2 or more variants; for example, *erm(A)* has two variants *erm(A)* and *erm(TR)*. Likewise for *erm(T)*, 17 variants of genes were recognized encoded by different transposons and plasmids[14]. Thus, each gene group has clonal nexuses, resulting, a priory, from exchanges of characters. Indeed, MLSB resistance in Cd-s strains of *Streptococci* carrying any version of *erm* gene is inducible to express clindamycin resistance, by the Er-r character. Besides *S. pyogenes*, other *Streptococcus* sp. such as, Group C and Group G species (*Streptococcus agalactia* and *Streptococcus pneumonniae*) also have *erm(A)*. Further, in the other species originally isolated from fermentation units, *Peptostreptococcus magnus* also was reported to have *erm(A)* gene[15], conferring it virulence along with drug resistance, as both characters are linked[7]. Conjugative transfer of the *erm(A)* gene from *erm(A)* positive isolates of *S. pyogenes* to Er-s strains of *S. pyogenes*, *E. faecalis* and *Listeria innocua* was recorded[16]. The other methylase gene, *erm(AM)* now called as *erm(B)*, coding resistance to MLSB antibiotics was associated with the constitutive, as well as the inducible MLSB phenotypes[16]. This bacterial pathogen was reported to be resistance to streptogramin A too[17]. Over the last 3 decades, N6 methyl transferase had been isolated from different bacterial species that cause DNA adenine methylation; similar methylation was recorded causing the methylation of the 23s rRNA in enterococci and *Staphylococci*, which have been involved in MLSB resistance with a lot of variances, named alphabetically from *erm(A)* up to *erm(Y)*[17]. Moreover, the gene *erm(A)* was detected late in *E. faecalis*, but was detected early in *S. pyogenes*[18]. Thus, these characters remain peripatetic among GP bacteria.

Our study corroborated similar studies from Korea, Belgium and 24 European Universal hospitals including Columbian hospitals[18-20]. Enterococci particularly, *E. faecalis* normally found in raw meat contaminated from fecal matter in slaughter house have a high level of MDR strains and, concomitantly contaminate surface/fresh water system too[21]. Intrinsic resistance of *E. faecalis* had been described to a group of antibiotics including the newly adopted daptomycin and vancomycin intended for GP bacteria[22]. In an Iranian study, the prevalence of strains resistant to glycopeptides, aminoglycosides and macrolides independently and with combinations in *E. faecalis* and *Enterococcus faecium* were described along with erythromycin resistance, *i.e.*, positive for *erm(B)* gene, in 45% and 41% of isolates, respectively. Further, both species had *erm(A)* gene at 5% isolates, but *erm(C)* gene was not detected. And clinical isolates of *E. faecalis* from India were reported having *erm(A)* and *erm(B)* genes[23]. In a study from Canada, it had been shown that 387 *E. faecalis* strains had a variety of combination of resistant genes on plasmid that caused resistance to a group of 18 antibiotics including bacitracin, chloramphenicol, flavomycin, quinupristin-dalfopristin and tylosin, mainly[21]. From China 117 *E. faecalis* isolates from swine yielded erythromycin resistance strains with 7 virulence genes, with resistance to tylosin and ciprofloxacin, in a large number[24]. As seen from Tehran, 12% strains were VRE consisting of 6% VRE *E. faecalis* and 22% *Enterococcus faecium*. Those were of the vanA phenotype[25]. Thus, erythromycin induced clindamycin resistant and multidrug resistant strains of *E. faecalis* are present universally, along with increasing levels of glycopeptide resistance particularly.

The antibiotic policy in the present era has been the root-cause of transforming both original harmless commensal groups, *Staphylococci* and enterococci to opportunistic pathogens, rendering them multidrug resistance, which frequently cause suppurative, enteric and UTIs[3]. Further, drug resistant characters are peripatetic across different groups of bacteria, even to phylogenetically distant ones by mobile genetic elements, transposons and plasmids, which mediate the exchange of genetic character. Among enterococci, streptococci and *Staphylococci*, the exchange of multidrug resistance should occur more readily, because these are GPs with the facultative mode of nutrition. Obviously, aggrandizement of multidrug resistant characters have rendered them the virulence character, as drug resistance begets virulence in bacteria[26]; and multidrug resistance renders unique survivability in the presence of an antibiotic stress, to which the bacterium wins over with the notoriety being directly proportionate to the extent of multi-resistance. Moreover, the geographic isolation mechanism has generated a group of sub-species within the GP bacterium, *E. faecalis*: *Enterococcus haemoperoxidus*, *Enterococcus moraviensis* and *Enterococcus rottae* with serological differences[27]. Furthermore, all these *E. faecalis* sub-species and other species of *Enterococcus* constitute a cohort of 27 sub-species, which are individually different from one another with cytological details, *i.e.*, cell wall chemistry and in their differential antibiotic resistant patterns[26]. Indeed, those create conundrums in food technology and havocs in public health, linked to endocarditis and comorbidities related to UTI[2].

Erythromycin induced clindamycin resistance of a considerable fraction of *E. faecalis* isolates would create a fear of epidemic, which could lead to boils, carbuncles, cystitis, pyelonephritis, bacteremia and acute comorbidities as blood stream infections. Moreover, it could be stated that, hospitalized patients, or patients who had used antibiotics within last 90 days have got an ample chance of picking of D-test positive *E. faecalis*. Vancomycin resistance plays a pivotal in public health sector, as this antibiotic is the coveted antibiotic in empiric therapy for GP bacteria. Clearly, the distribution of *MLSB* gene in erythromycin resistance strain is pivotal in the expression of clindamycin resistance induction in Er-r and Cd-s strains. Further, it could be stated that the D-test protocol should be followed with clinical samples

in hospitals, where most GP bacteria are found MDR in higher percentages.

Conflict of interest statement

We declare that we have no conflict of interest.

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