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Prevalence and genetic profiling of tetracycline resistance (*Tet-R*) genes and transposable element (*Tn916*) in environmental *Enterococcus* species

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ABSTRACT

Resistance against antimicrobial agents in enterococci is a global concern that not only challenges infection therapy but also make them reservoir of antibiotic resistance in human and animal alike. This study was conducted to establish tetracycline resistance profiles, prevalence of *tet* genes and transposable element (*Tn916*) in enterococcal soil and clinical isolates. Enterococci (n = 1210) from different environmental niche were collected and subjected to molecular identification. In total, 361 isolates showed tetracycline resistance at the breakpoint of 32 μg ml^{-1}. MICs (32–512 μg ml^{-1}) were established by both agar and micro-broth dilution methods. Soil isolates (n = 76) were further investigated for *Tet* genes (*tet-A, C, K, L, M, S, O*) and *Tn916*. Major resistance was observed in *E. faecium* 67% followed by *E. faecalis* 22%, *E. hirae* 8% and *E. casseliflavus* 2.6%. Results revealed that *tet(L)* was more frequently found in *E. faecium* 74.5%, while *tet(M)* was in high prevalence in *E. faecalis* 82.3%. *Tn916* was detected in both clinical and soil isolates (i.e. 43.3% and 19.7%, respectively). RAPD-PCR analysis showed high diversity among the investigated isolates. Cumulatively, our results revealed high-level tetracycline resistance and the presence of multiple *Tet* genes and transposable element *Tn916* in enterococci.

1. Introduction

The development, proliferation and dissemination of antibiotic resistance in human pathogens is a major health concern which have consequently increased the economic burden, morbidity and mortality rate [1]. Enterococci (comes under the umbrella of lactic acid bacteria (LAB)) are Gram positive, facultative anaerobes, ovoid shaped cocci. Their primary niche is digestive tract of warm blooded animals including humans [2]. They intrinsically possess virulent traits as well as antibiotic resistance genes and may acquire multi-drug resistance (MDR) through horizontal gene transfer (HGT) that has played profound role in the acquisition, accumulation, and dissemination of extra-chromosomal DNA i.e. plasmids, transposons and integerons harbouring virulence or antibiotic resistance genes [3]. These genetic changes contribute to survival advantages under critically stressed environmental conditions enabling non-pathogenic gut commensal to become opportunistic, pathogenic and antibiotic resistant super bugs ultimately raising therapeutic challenges [4]. Selective pressure and genetic transfers are main sources of development of antibiotic resistance among microorganisms and transmission of resistant strains is facilitated by the existence of ecological reservoirs (human, animal, and environment) [5]. Those genetic changes have transformed these gut normal bacteria to virulent and antibiotic resistant pathogens involved in hospital acquired infections which pose serious threat to health care systems [6]. Enterococci are ranked as second common cause of nosocomial infections worldwide [7]. *E. faecalis* is the most common species associated with clinical infections, while *E. faecium* poses the highest antibiotic resistance threat [8,9]. In humans, they are involved in life threatening infections such as meningitis, dental infections like gingivitis, periodontitis, teeth with failed endodontics, infected root canals, ear infections, endophthamilitis, paranasal sinuses, burns and wounds abscess, surgical wound infection, urinary tract infections, hepatobiliary sepsis, endocarditis, neonatal sepsis, catheter related infections, and bacteraemia that could be lethal in immuno–compromised patients [10,11]. Enterococci can easily be disseminated into extra-enteric environments thus polluting water, soil and air. Thus, their

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presence in the environment is considered as an indication of faecal contamination [12,13].

Tetracycline (Tet), discovered in 1940's are broad spectrum antibiotics that exhibit activity against both Gram-positive and Gram-negative bacteria. Tetracycline are bacteriostatic, polyketide antibiotics produced by the *Streptomyces* genus of *Actinobacteria.* Reduced side effects and cheaper cost have led to extensive use of tetracycline in treatment of many human as well as animal infections [14]. They have also been used as anti-malarial agents against malaria caused by *Plasmodium falciparum* which is resistant to mefloquine [15]. They are also used in animal feed as growth promoters [16]. Tetracycline binds reversibly to the 30S subunit of the bacterial ribosome, blocking the binding of aminoacyl–tRNA to the acceptor site on the mRNA–ribosome complex and consequently disrupting the protein synthesis [17]. Enterococci may acquire different mechanisms of tetracycline resistance through *tet* genes encoding proteins with diverse functions. The most common are (a) Efflux of drug and (b) Ribosomal protection. Efflux pumps are membrane-associated proteins (45 kDa) which export tetracycline from the cell. Export of tetracycline reduces the intracellular drug concentration and thus protect the ribosome from halting of translation. Ribosomal protection proteins (72 kDa) are in cytoplasm, bind to the ribosome and altering its conformation that prevents tetracycline binding to ribosome, while protein synthesis is continued [18]. Genes involved in efflux mechanism include *tet(K)* and *tet(L)*, while *tet(M)*, *tet(O)*, *tet(Q)* *tet(S)*, *tet(T)* and *tet(W)* are reported to be involved in ribosomal protection in enterococci [19].

Main factor responsible for very high resistance to tetracycline is might be the natural synthesis of tetracycline by *Actinomycetes*. Soil is a natural reservoir of micro–biota and production of tetracycline by some microbes like *Actinomycetes* and extensive use of antibiotics in agriculture (as a growth promoter to increase crop yield) make soil (both bulk soil (BS) and agriculture soil (AS)) a natural reservoir of multiple drug resistant (MDR) bacteria. This situation is quite alarming for human and animal health alike and poses serious threat to the ecosystem [20].

There is no baseline data regarding enterococci as well as tetracycline resistance profile available so far which can reflect the mortality rate and economic burden faced by Pakistan, where public health is already compromised due to poverty and lack of awareness regarding hygiene and proper use of antibiotics. Thus, the present study is designed and persuaded specifically on tetracycline resistant enterococci (TRE) from the environment. Large number of enterococcal isolates (n = 1210) were collected from each niche of Karachi, the capital of Sindh and termed as Beta world city. It is the most populous city due to Pakistan's premier industrial and financial centre [21]. Molecular identification and genetic relatedness of the isolated *Enterococcus* species, tetracycline resistance profiling, prevalence of *tet* genes, and transposable element *Tn916* (which is responsible for the transposition of *tet* genes) in soil and clinical isolates have been conducted.

2. Materials and methods

2.1. Isolation and identification of Enterococci

Samples from different environmental sources (including sewage, soil, birds/poultry manure, animal manure) as well as clinical isolates were collected during the study period (2014–2015) from Karachi city. Samples were obtained aseptically in sterile glass screw cap tubes. For the isolation of enterococci from sewage and manure samples, protocol was followed as described in an earlier study by Lanthier et al. [22]. While enterococci isolated from poultry manure and agriculture soil was performed using the protocols recently described [23,24]. Bulk soil isolates were included from a recent study conducted by us [24]. Briefly, 100 μL of 1:10 saline diluted samples were inoculated and pre-incubated for 8–12 h at 45 °C in 900 μL BHI broth containing 6.5% (w/v) NaCl. A loop full of 12 h culture was used to purify enterococcal colonies by streaking on bile-aesculine azide agar plates (Oxoid, UK). After incubation at 37 °C for 24 h, pure colonies exhibiting black-brown zone of aesculin hydrolysis (aesculetin) were considered as presumptive enterococci. Further confirmation was conducted by the reduction of 0.1% triphenyl tetrazolium chloride (TTC) in Slanetz and Bartley agar (SBA, Oxoid, UK).

2.2. Molecular identification of presumptive enterococcal isolates

Genus and species level identification of the presumptive enterococci was achieved by multiplex–PCR method originally described by Jackson et al. [25], targeting *16S rRNA* (ribosomal) and *sodA* (superoxide dismutase) genes (respectively) with slight modifications as recently described by Ali et al. [24]. All PCR was performed using thermal Master cycler (*ProS* Eppendorf, Germany).

2.3. Tetracycline resistance phenotypes

All identified enterococcal isolates were screened for tetracycline (Amresco, USA) resistance at break point of 32 μg ml^{-1} by agar dilution method using Mueller-Hinton agar (Oxoid, UK) as per Clinical and Laboratory Standards Institute (CLSI) guidelines [26].

2.4. Determination of MICs using agar and micro-broth dilution methods

Minimum inhibitory concentrations (MICs) of tetracycline 32–512 μg ml^{-1} against enterococcal isolates were determined by agar dilution method using Mueller–Hinton agar (Oxoid, UK) [27]. In addition, MICs of the representative soil isolates from each tetracycline resistant group were further complemented by micro–broth dilution method using sterile 96 well microtiter plates (Corning, NY, USA) by the protocol described elsewhere [28]. Bacterial cultures used in MICs determination were equivalent in turbidity to 0.5 McFarland turbidity standard. *E. faecalis* ATCC 29212 and *E. faecium* NA283 (in house control) were used as positive and negative controls, respectively.

2.5. RAPD–PCR

Genetic relatedness and variation was established by Randomly Amplified Polymorphic DNA (RAPD) PCR using specific oligonucleotide primer M13 (Table 1) as described by Muñoz-Atienza et al. [29]. Briefly, PCR was performed in a total volume of 20 μL comprising of 10 μL of 2 X Dream *Taq* PCR buffer (Thermo Scientific, USA), 16 μM of primer M13, 1.0 mM of $MgCl_2$, 10% DMSO (Sigma-Aldrich, USA), and 2.5 μL of gDNA (bacterial lysate). The thermal programme used was as follows: initial denaturation at 95 °C for 5 min, 35 cycles of amplification (denaturation at 90 °C for 30 s, annealing at 40 °C for 1 min, extension at 65 °C for 8 min), and a final extension at 68 °C for 16 min. The American Type Culture Collection (ATCC) strains of *E. hirae* ATCC 8043, *E. faecium* ATCC 6569, *E. casseliflavus* ATCC 25788 and *E. faecalis* ATCC 29212 (Oxoid, UK) were used as positive controls. 1 kb and ultralow molecular weight DNA ladders (Fermentas, USA) were used as size standards. PCR products were subjected to 2% agarose gel for 5 h at 60 V (~25 °C). The resulting patterns produced from RAPD–PCR were interpreted after constructing dendogram using UPGMA (unweighted–pair group method with arithmetic mean) and similarity based on Dice's coefficient analysed with *GelJ* v.2.0 software [30].