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DNA AND PROTEIN FINGERPRINTING OF BACTERIOCIN PRODUCING *ENTEROCOCCI* OF CLINICAL ORIGIN

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ABSTRACT

Introduction: *Enterococcus* is one of the most common etiological agents of nosocomial infections with multiple clinical strains that produce bacteriocins. This study aims to explore the genomic and proteomic diversity of the bacteriocinogenic and non-bacteriocinogenic clinical *Enterococci.*

Methods: Stab overlay and cross streak methods were used to identify bacteriocin producing *Enterococci*. Bacteriocin producers and selected non-producers were taxonomically identified by 16S rDNA sequencing. Genomic variations of the isolates were explored by randomly amplified polymorphic DNA (RAPD-PCR), whereas sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was employed to discern the proteomic diversity of the isolates.

Results: Out of 109 clinical isolates screened, 9 and 4 were respectively found to be bacteriocin producers and non-producers. Of 13 selected isolates, 8 were identified as *Enterococcus faecalis* and 3 were identified as *Enterococcus faecium*. Two isolates, SMN14 and SMN17, were failed to amplify by universal primers of 16S rDNA gene. RAPD analyses showed that out of 6 arbitrary primers, 3 were able to successfully resolve the genetic variations present amongst the isolates of *Enterococcus faecalis* or *Enterococcus faecium*. Consistently, SDS-PAGE of total bacterial lysate not only demonstrated the total protein expressional differences amongst the selected isolates but also distinguish bacteriocin producers from non-producers.

Conclusions: Our findings show that simple assays like SDS-PAGE and RAPD may not only augment taxonomic resolution of *Enterococci* but also points to their metabolic potential like bacteriocin production. Therefore, such approaches could further be exploited for epidemiological investigations of *Enterococci* and potentially other bacterial pathogens. Nevertheless, large scale studies are warranted in this regard.

Keywords: Bacteriocin; *Enterococci*; Fingerprinting; RAPD; SDS-PAGE.

INTRODUCTION

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by variety of bacteria, and *Enterococcus* are no exception in this regard (Riley and Wertz, 2002; Ness *et al.*, 2014). Both experimental and bioinformatic based genome mining approaches have been exploited to explore bacteriocinogenic potential of bacteria (Hammami *et al.*, 2010; Jabeen *et al.*, 2014). However, strain identification of the bacteriocin producer still lacks resolution by conventional microbiological and biochemical means. Molecular identification based on DNA sequencing of housekeeping genes like 16S rDNA may resolve the species of the isolate, however, may not be suitable to delineate intra species differences (Fox *et al.*, 1992). To overcome this caveat, approaches like Pulse Field Gel Electrophoresis (PFGE), Random Amplification of Polymorphic DNA (RAPD) PCR and Ribotyping have been used for several bacterial species including *Enterococci* (Banerjee, 2013; Werner *et al.*, 2015). This study provides the first genetic and proteomic profiling of bacteriocinogenic clinical isolates of *Enterococcus* by RAPD and SDS-PAGE to represent their genetic diversity.

MATERIALS AND METHODS

Isolation of Enterococci: The study in its entirety is empirical and did not involve any humans and/or animal subjects, therefore exempted from conventional ethical review. In total 109 enterococcal isolates were obtained from Dow Diagnostic Research Reference Laboratory (DDRRL), Karachi and coded as SMN (S: Sanya; M: Mushtaq; N: Nusrat).

Screening for Bacteriocinogenic Enterococci: After conventional microbiological verification, all enterococcal isolates were screened for bacteriocin production against each other by employing cross streak and stab overlay methods. Briefly, in cross streak method, plates of Brain heart infusion agar (BHI) were inoculated with the enterococcal strain as a vertical streak across the surface of plate and incubated at 37°C for overnight, next day to kill the

producer strain, growth of bacteria removed and plates were exposed with chloroform vapours using whatman filter paper for 15mins, then sensitive strain were cross streaked perpendicular to each of the producer strain followed by overnight incubation at 37°C, next day zone of inhibition were observed (Pugsley and Oudega, 1987). In case of stab over lay method BHI agar were first stabbed with a producer enterococcal strain and incubated at 37°C for overnight. Later on, plates were exposed to chloroform vapours for 15mins. Subsequently, 10µL of 2hrs pre-incubated culture of sensitive enterococcal strain was mixed with the 0.6% BHI (soft agar) and the suspension was overlaid on the agar plates, followed by incubation at 37°C for overnight and then examined for clear zone of inhibition around the stab of producer enterococcal strain. (Cooper and James, 1984).

Taxonomic Identification: Selected bacteriocin producer and sensitive bacterial isolates were taxonomically identified by amplification (colony PCR) and 16S rDNA gene sequencing using universal primers (Table 1).

Table 1: Sequences of primers used in the present study.

Primers	Direction	Sequences (5'-3')
FD1 16Sr DNA	Forward	AGAGTTTGATCCTGGCTCAG
RP1 16Sr DNA	Reverse	CGGTTACCTTGTTACGACTT
pol-1	----	GTCGTCCATGTA
pol-2	----	GTCATCCATGTA
pol-3	----	ATCGTCCATGTA
pol-4	----	GTCGTCCATATA
ran-3	----	GTTGCGATCC
ran-4	----	AGGTGACCGT

The PCR amplification was conducted with an initial denaturation at 95°C for 10mins followed by 35 cycles involving 95°C for 30sec (denaturation), 50 °C for 1mins (annealing), 72°C for 1min (extension) and final extension at 72°C for 10min in a 35 µL master mix containing 1x KCl PCR buffer, 4m M of $MgCl_2$, 2mM of dNTPs, 7U of Taq DNA polymerase, 2µM forward and reverse primers each. A very small portion of bacterial colony was added in the master mix. After PCR amplification, the amplicons were subjected to DNA sequencing at Macrogen, South Korea. Final species identification is based on non-redundant, nucleotide Basic Local Alignment Search Tool (BLAST), followed by sequence alignment using CLUSTALW with standard reference strain sequence of *E. faecalis* (strain V583) and *E. faecium* (strain DO) (Larkin *et al.,* 2007). The cladogram was constructed by the maximum likelihood method (MLH) with 1000 bootstraps replicates employing Hasegawa-Kishino Yano (HKY) evolutionary model using MEG A6 (Tamura *et al.,* 2013).

DNA Finger Printing: Genomic variation was explored among enterococcal isolates by RAPD-PCR using six random primers (pol-1 to pol-4, ran-3 and ran-4) (Table 1). The PCR amplification was conducted with initial denaturation at 94°C for 10mins followed by 45 cycles of 94°C for 30sec (denaturation), 31°C for 30sec (annealing) and 72 °C for 1min (extension), a final extension was done at 72°C for 4min in a 25µL master mix of 1x KCl PCR buffer, 4mM of $MgCl_2$, 2mM of dNTPs, 15U of Taq DNA polymerase, 4µM of each primer. Finally, a very small portion of bacterial colony was added in the master mix. PCR amplicons were electrophoresed in 2% agarose gel (Molequle-on) in 1xTAE buffer and amplified fragments were visualized and photographed under UV trans-illuminator. For the bands comparison gel images were analyzed by Gel Analyzer. Finally, cladogram was constructed by Neighbour Joining method using Free Tree and for final annotation, Fig Tree v1.4.2 was employed (Hampl *et al.,* 2001).

Protein Finger Printing: Proteomic diversity between enterococcal sensitive and producer strains was compared by electrophoresing the total proteome in gradient (4-20%) SDS polyacrylamide gel. The bacterial cell pellet was obtained from the overnight culture grown in heart infusion broth by centrifugation at 9600g. Bacterial cell pellet (0.1 gm) was suspended in 500µL bug buster (MERCK) and incubated at room temperature for 45mins at 50rpm. Subsequently, the soluble proteins were separated by centrifugation at 9600g for 15mins at 20-25°C. Supernatant was eluted and bacterial lysate suspension was electrophoresed on gradient (4-20%) SDS-PAGE and bands were visualized by coomassie blue staining. The protein gel image was analysed using Gel Analyzer. The cladogram was generated by neighbour-joining method (NJ) with 1000 bootstrap replicates using Free Tree and visualized in Fig Tree v1.4.2 (Hampl *et al.,* 2001).

RESULT

Screening of Bacetriocin Production is Dependent on Assay System: In total 109 enterococcal clinical isolates were collected from DDRRL, which were further confirmed as *Enterococci*, based on dark brown to black coloured colonies on bile aesculin agar (Figure 1A). Screening of bacteriocin producers revealed that around 10% isolates of *Enterococci* were found bacteriocinogenic. In comparison to 8 isolates found in cross streak method, stab overlay represented 13 isolates as bacteriocinogenic (Figure 1B).

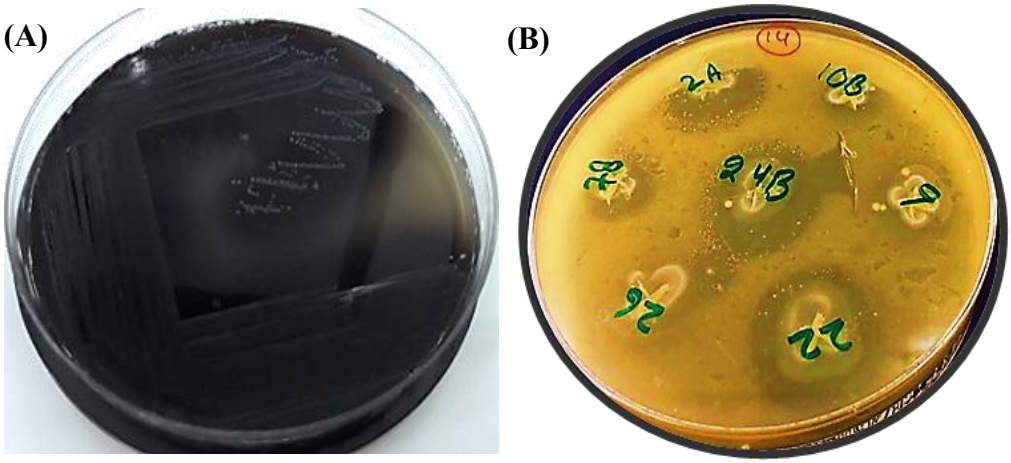

Figure 1: Isolation and screening of bacteriocin producers. (A) Characteristics pin pointed black colour colonies of *Enterococcus spp.* on bile aesculin agar. (B) Stab-over lay method for bacteriocin detection, zone of inhibition represent antibacterial activity of bacteriocin produced by stabbed isolate against the over layered strain.

Except 2 isolates, SMN14 and SMN17, 16S rDNA gene was amplified for all other isolates (Figure 2 A) and the respective gene was successfully sequenced (Figure 2B). Out of the 13 selected isolates, 8 were identified as *E. faecalis*, whereas 3 were found *E. faecium* (Figure 2C). The sequences were accessioned by NCBI nucleotide database as MG9 66442, MG966443 and MG975085-MG975093.

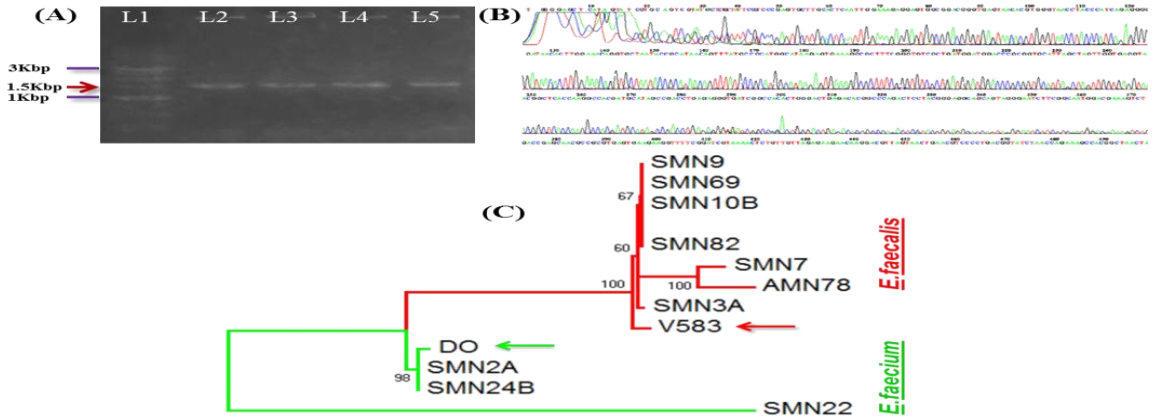

Figure 2: Molecular identification of enterococcal isolates. (A) Representative gel of 16S rDNA amplification (L2-L5) by colony PCR, where the respective gene amplification (~1500bp) is shown by red arrow. L1 contain DNA ladder of 1Kbp. (B) Representative electropherogram of DNA sequence of 16S rDNA of *E. faecalis*, where base calls are represented by different colours. (C) Cladogram constructed on the basis of 16S rDNA sequences, where reference strains of *E. faecalis* and *E. faecium* are indicated by red and green arrows, respectively.

Bacteirocin producers could be differentiated on the basis of genomic finger printing: In RAPD analysis, three arbitrary primers, pol-3, ran-3, and ran-4, were able to discern the genomic variability of the strains conspicuously, as shown in the respective gels and cladograms (Figure 3). In comparison, remaining three primers, pol-1, pol-2 and pol-4, were able to demonstrate the genomic variations in most of the isolates except SMN2A, SMN 22, SMN3A and SMN69 in case of pol-1, SMN22 and SMN24B in case of pol-2 and SMN78 and SMN82, when amplification was carried out using pol-4 as primer (Figure 3).

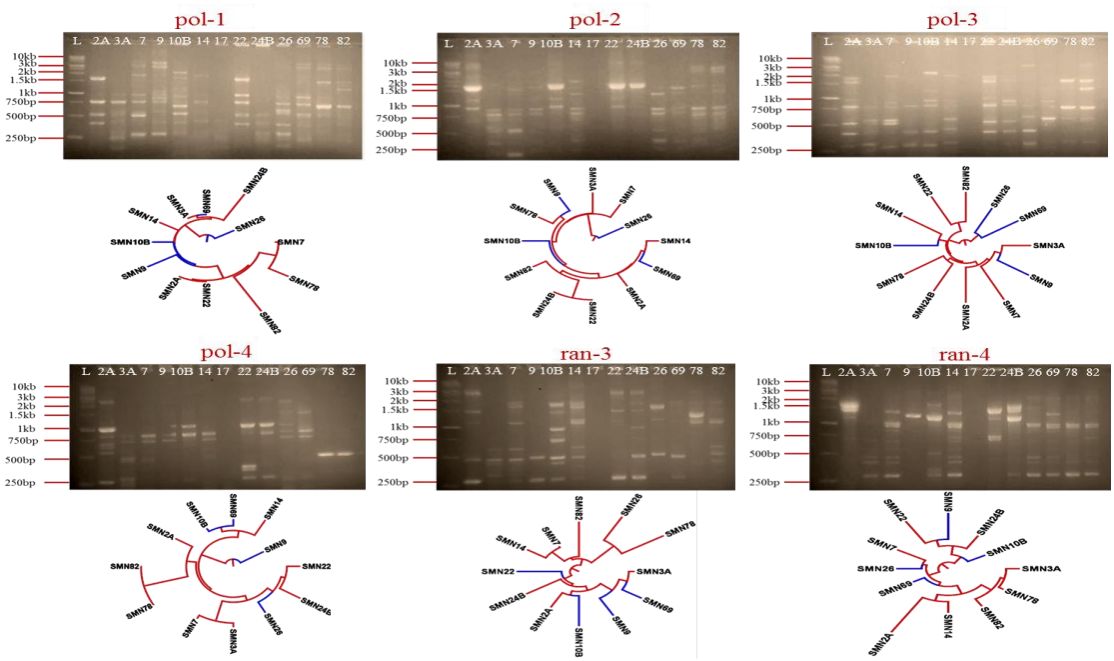

Figure 3: RAPD-DNA fingerprinting. RAPD profiling of enterococcal strains by using pol-1, pol-2, pol-3, pol-4, ran-3, ran-4 primers (labelled above). First lane (left side) of each gel contains 1Kbp DNA ladder with different band sizes annotated. Remaining lanes of each gel shows genomic DNA amplification of respective strains by respective primer. At the bottom of the each gel their respective cladogram is shown, where red lines indicates bacteriocin producers and blue is for non-producers strains.

Bacteriocin producers could be differentiated on the basis of protein finger printing: Protein separation by gradient (4-20%) SDS-PAGE showed differential protein expression pattern between the bacteriocinogenic and non-bacteriocinogenic enterococcal isolates (Figure 4). Bacteriocinogenic strains were found to be more prolific in terms of magnitude and diversity of the protein expression compared to non-bacteriocinogenic strains. This is being more clarified in the cladogram constructed based on of bands (protein) pattern, suggesting the potential link between the overall metabolic activities of the strains with the bacteriocin production (Figure 4).

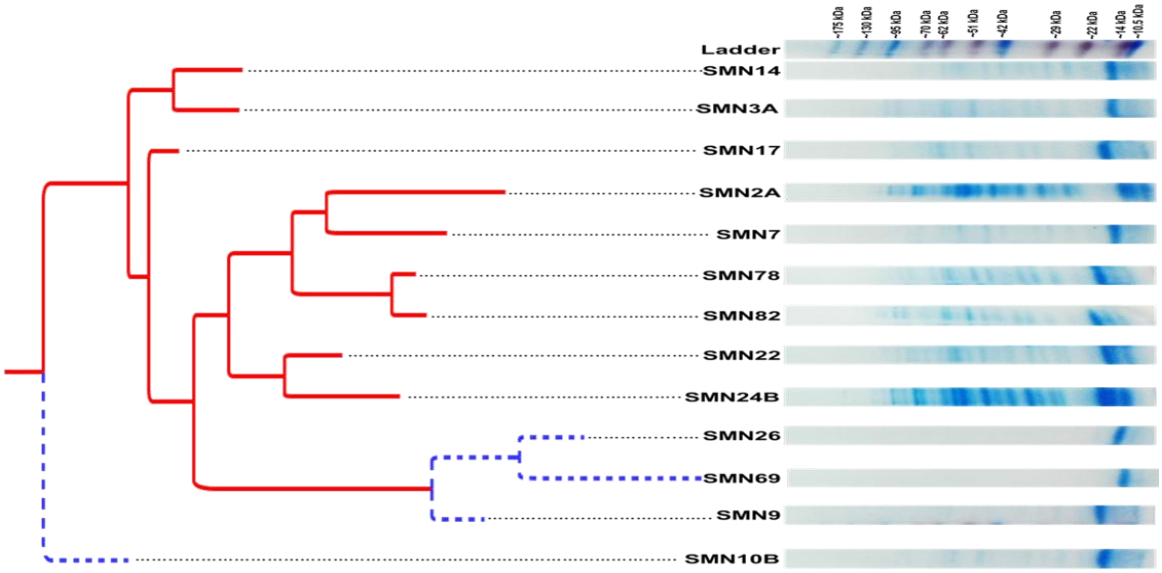

Figure 4: Protein profiling. Neighbour joining tree was constructed, based on molecular weights and number of proteins expressed in different enterococcal strains, collected from respective bacterial lysate. Where red lines represent bacteriocin producers while blue represent non-producer strains. Corresponding to the each line expression of protein in (4-20% gradient) SDS-PAGE is shown.

DISCUSSION

E. faecium and *E. faecalis*, are recognized as one of the most important causes of nosocomial infections such as urinary tract infections, neonatal sepsis, endocarditis, hepatobiliary sepsis, surgical wound infections and bacteremia (Selleck and Van Tyne, 2019). Consistently, in our study, all screened clinical isolates were identified as *E. faecalis* and *E. faecium*. Clinically isolated strains of both *E. faecalis* and *E. faecium* have exhibited resistance against several antibiotics including vancomycin (Arias and Murray, 2012). Bacteriocins, antimicrobial peptides produced by bacteria, have been suggested and shown as a possible alternative to antibiotics (Cotter *et al.,* 2013). Isolates of several species of *Enterococcus, E. avium, E. durans, E. hirae, E. faecium, E. faecalis* and *E. mundtii* species are recognized as potent producers of bacteriocin (Ness *et al.,* 2014; Qiao *et al.,* 2020). In our study 8 and 13 isolates were found to be bacteriocin producers by cross streak and stab over lay method, respectively. This suggests that stab overlay method is relatively more sensitive for the detection of bacteriocin producer. This is in line with the earlier study conducted by Choeisoongnern *et al., (2019)* showing variation in identification of bacteriocin producers due to the method of screening. It has been suggested that nearly 99% of the bacteria are bacteriocin producers, however, their appropriate identification is the function of availability of the suitable sensitive strains (Riley and Wertz, 2002). The low frequency of the bacteriocin producers in the present study could be due to the screening against the more naturally resistant strains. In addition, comparisons has been previously carried out for the potential of bacteriocin production between environmental and clinical isolates, demonstrating the former tend to have more ability to produce novel bacteriocins (Birri *et al.,* 2013).

Conventional microbiological, biochemical and molecular approaches have been widely employed in the species identification of the enterococcal isolates. For example, 16S rDNA gene sequencing is a reliable and well-established approach, used for enterococcal identification at least at the species level (Fox *et al.,* 1992). Similarly, in our studies, isolates were identified as either *E. faecalis* or *E. faecium* by 16S rDNA sequencing. However, due to the sequence conservation in the housekeeping genes, it often fails to resolve intra-species variations (Fox *et al.,* 1992). To overcome this caveat approaches like RAPD-PCR, Pulse Field Gel Electrophoresis (PFGE), Multi Locus Sequence Typing (MLST), repetitive PCR typing, plasmid profiling, Amplified Fragment Length Polymorphism (AFLP), Multiple Locus Variable Number of Tandem Repeat Analysis (MLVA) and ribotyping are often employed for genotyping of bacterial pathogens including *Enterococci* (Banerjee, 2013; Werner *et al.,* 2015). Consistent to these observations, in the present study, genomic diversity of isolates were discerned using 3 out of 6 primers by RAPD-PCR. Since RAPD-PCR based genotyping could well be affected by the sequence of the arbitrary primers (Caetano-Anolles, 1993), therefore, sequences of the primers like pol-3, ran-3, and ran-4 could be used in order to distinguish genomic variations in the enterococcal strains (Figure 3). Besides there are several advantages associated with the approach including: usage of non-radioactive primers (oligonucleotide probes), procedural simplicity, cost effectiveness, less time consumption and does not require any prior information regarding genomic data (Kumar and Gurusubramanian, 2011). With the availability of Next Generation Sequencing (NGS), in the recent times, microbiota of different niches such as clinical, environmental or food related, have been identified by whole genome sequencing (Cao *et al.,* 2017; Ge, 2017; Weinstock, 2012). Therefore, this provides another tool for further differentiation between bacteriocin producers and non-producers. In this connection, bacteriocin identifier tools of bioinformatics such as BAGEL and ANTISMASH could also be exploited (van Heel *et al*., 2013; Blin *et al*., 2016).

Protein profiling of complete cellular lysate of an organism (by SDS-PAGE) has been shown its utility in bacterial classification or strain differentiation. Perez *et al., (2000)* used the protein pro-filing strategy to discriminate the lactic acid bacteria at species and sub-species level. In the current study, SDS-PAGE profile clearly differentiated the selected enterococcal isolates. Moreover, it is also able to distinguish between bacteriocinogenic and non-bacteriocinogenic strains (Figure 4). This points to the potential link between the overall metabolic activities of the organism with its bacteriocin production.

Although both RAPD-PCR and SDS-PAGE profiling able to distinguish isolates based on their genomic and proteomic variations, respectively, however, the study and/approach hold some limitations. For instance, bacteriocin production could further be verified from cell free supernatant. Ideally, equal number of bacteriocin producers and non-producers, should be incorporated in the study, however, given that 99% of the bacterial isolates are bacteriocin producers, therefore this may not be practically achievable (Riley and Wertz, 2002). Besides RAPD-PCR based genotyping and SDS-PAGE based protein profiling could further be

verified by PFGE and 2D protein gel electrophoresis, respectively.

CONCLUSIONS

The study is the first in connection to distinguish between bacteriocin producers and non-producers concerning to their genomic and proteomic variations. It forms the baseline for a relatively large scale study to explore the possibilities for its application in the identification of bacteriocin producers and molecular epidemiological studies of *Enterococci*.

CONFLICT OF INTEREST: None

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