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Research Article

SCREENING OF FUNGAL ISOLATE AGAINST CLINICAL ISOLATES OF *STAPHYLOCOCCUS AUREUS* BY CO-CULTURE ASSAY FOR NOVEL ANTIMICROBIAL COMPOUNDS

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ABSTRACT

Asia is among the regions with the highest prevalence rates of healthcare-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) and community-associated methicillin-resistant *S. aureus* (CA-MRSA) in the world. To overcome some of these severe multi-drug resistant pathogens, eukaryotes are better source of diverse compounds, in which fungi serves as second largest group after plants, producing bioactive secondary metabolites. In the present study using co-culture assay method, the fungal isolates were screened against clinical isolates of *Staphylococcus aureus* (Gram positive coccoid bacteria) that is known to cause simple skin infections to life threatening septiceamia. Clinically they are known to show an alarming rate of resistance against multiple antibiotics (multi-drug resistance). Twelve, *S. aureus* isolates, screened for multi-drug resistance were procured from KIMS Medical College and Hospital, Hubballi Karnataka, India. To assess the resistance pattern of these clinical isolates, antibiogram assay was performed by Kirby Bauer's method. The antibiotics used for this assay were penicillin, clindamycin, methicillin, vancomycin and lincomycin. The zone of inhibition was measured by the reference standards of CLSI (Clinical Laboratory Standards Institute) chart. Our results infer varying resistance pattern for penicillin 83%, clindamycin 2%, methicillin 100%, vancomycin 8.3% and lincomycin 8.3%. To verify its antagonistic property, these *S. aureus* isolates were co-cultured with fungi. Co-culture assay has been observed as one of the efficient tools to evaluate the metabolite production ability and antibacterial efficacy of the fungal isolates. Results were evaluated on the basis of zone-of contact inhibition, distance inhibition, zone-line inhibition and overgrowth inhibition. Our results out of 12 interactions revealed, 05-contact inhibition, 03-overgrowth inhibition, 02-distance and 02-zone line inhibition.

Keywords: Fungal metabolite, *S. aureus*, Multi-drug resistance, Antibiogram, Co-culture assay

1. INTRODUCTION

Multi-drug resistance in bacteria is a process of non-responsiveness to antibiotics due to production of enzymes that are on a single R plasmid, derived by mechanisms provided by transposons, integrons, and ISCR elements [1]. From the epidemiological perspective, multi-drug resistant organisms (MDROs) are among the microorganisms (predominantly bacteria) that show resistance to one or more class/es of antibiotics [2]. Asia is among the regions with the highest prevalence rates of healthcare-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) and community-associated methicillin-resistant *S. aureus* (CA-MRSA) in the world [3]. *S. aureus* is a Gram positive coccoid bacteria belonging to family firmicutes. Many of these strains cause serious nosocomial infections and are resistant to virtually all the useful antibiotics, including methicillin, cephalosporins and even the family of polymyxins and cationic polypeptides [4]. Some clinical strains of *S. aureus* have even developed resistance to streptomycin, neomycin and vancomycin [5, 6]. Majority of current antibiotics are of prokaryotic origin, majorly from actinomycetes and there are multidrug resistant organisms against these antibiotics too [7, 8]. In eukaryotes, fungi are better source of diverse and complex secondary metabolites, the macroscopic and microscopic fungi are the most significant producers of bioactive metabolites. These significantly contribute to an approximately 8600, representing 38% of all the microbial products [9]. Thus fungi can be source for effective secondary metabolites as antibiotics against multi-drug resistant pathogens [10, 11]. In this study, we propose that using antibiogram assay for assessment of multi-drug resistance followed by co-culture assay for antibiotic producing ability of fungal isolates against multi-drug resistant *S. aureus*.

2. MATERIAL AND METHODS

2.1. Fungi isolation

Soil samples were collected from unique habitat with predicted microbial diversity, in and around Karnatak University Campus, Dharwad. Samples were carried aseptically in polythene sample bags to the laboratory followed by serial dilution, plating on Potato Dextrose Agar (PDA) and Sabouraud's Dextrose Agar (SDA) media. Using 10^{-1}, 10^{-2} and 10^{-3} dilutions, uniform surface plating was done by using a spreader. The plates were incubated at room temperature (28-32 °C) for 48-72 hrs. Isolated colonies were picked and were sub-cultured, pure cultures were preserved for further use at 2-8 °C. Twelve sample of *S. aureus* clinical isolates procured from KIMS, Hubballi (Karnataka Institute of Medical Sciences) isolated from different medical samples, were subjected to antibiotic sensitivity assay to verify their multi-drug resistance pattern. The isolated fungi was screened for secondary metabolite producing ability by co-culture assay with respect to the activity against the multi-drug resistant *S. aureus* [12].

2.2. Antibiotic assay

Antibiotic assay was performed according to Kirby-Bauer's antibiotic susceptibility test and evaluated by Clinical Laboratory Standards Institute [13]. Kirby-Bauer's antibiotic susceptibility test was developed by Kirby and Bauer in 1950 and standardized by WHO in 1961 [14]. This test was conducted to determine the resistance or sensitivity pattern of pathogenic bacterial isolates of *S. aureus* to prescribed antibiotics. The presence or absence of an inhibitory area around the antibiotic disc identifies the bacterial sensitivity to the drug. The bacterium (*S. aureus* isolate) was swab inoculated on the Mueller-Hinton agar plates and the antibiotic discs (penicillin, clindamycin, methicillin, vancomycin and lincomycin) were placed on top of inoculated agar media [4]. The antibiotics diffuse from the disc into the inoculated agar media in decreasing concentration outwards in circular pattern from the antibiotic disc. If the organism is killed or growth is inhibited by the concentration of the antibiotic that diffuse from the filter disc, there will be a clear zone around the disc. This measure of zone (in mm) of inhibition leads to understand the MIC-minimum inhibitory concentration values of antibiotics with suitable concentration against *S. aureus*. The zone sizes were measured (in mm) and compared to the standard Clinical and Laboratory Standards Institute chart [8] that evaluates the results of the organism showing sensitive, resistant or intermediate activity against that particular antibiotic concentration.

2.3. Co-culture assay

Co-culture assay has been applied to study diverse issues, one among it is induction of pharmaceutically interesting secondary metabolites [8, 12, 15]. The co-existence of several microorganisms that share the same niche can affect the organism's growth, adaptation, patterns, morphology and developmental patterns [16, 17] as well as their ability to synthesize proteins and secondary metabolites. The best known example of bacterium and fungal interactions was the accidental discovery of penicillin. It was an unintended co-culture (contamination) of *Staphylococcus* spp. with *Penicillium* sp. in 1928 by Sir Alexander Fleming [18]. This lead to the understanding of the process of antagonistic activity by one organism or the other by virtue of first organism producing a secondary metabolite to inhibit the other organism's growth. The same activity is being tried and mimicked in the co-culture assay to produce an isolate using four different criteria zone-of contact inhibition, distance inhibition, zone-line inhibition and overgrowth inhibition.

3. RESULTS

3.1. Isolation of fungi

The soil samples yielded 46 pure fungal cultures, out of which 20 diverse fungal isolates (Fig.1) were used for screening of the antimicrobial metabolites. The organisms were sub-cultured and confirmed to be pure and further were stored in refrigerated condition, in duplicates for further use.

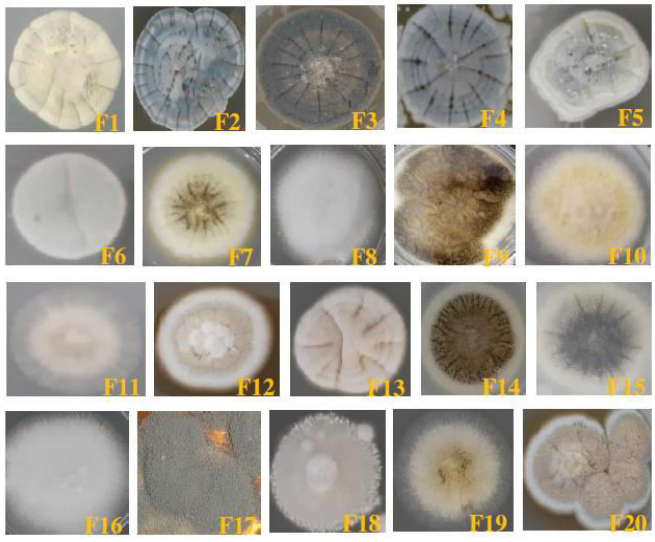

Fig. 1: Fungal isolates obtained during isolation from soil samples

3.2. Antimicrobial assay

Antimicrobial assay conducted against all 12 isolates obtained from KIMS, Hubballi showed different zone of inhibition that was observed with different *S. aureus* isolates.

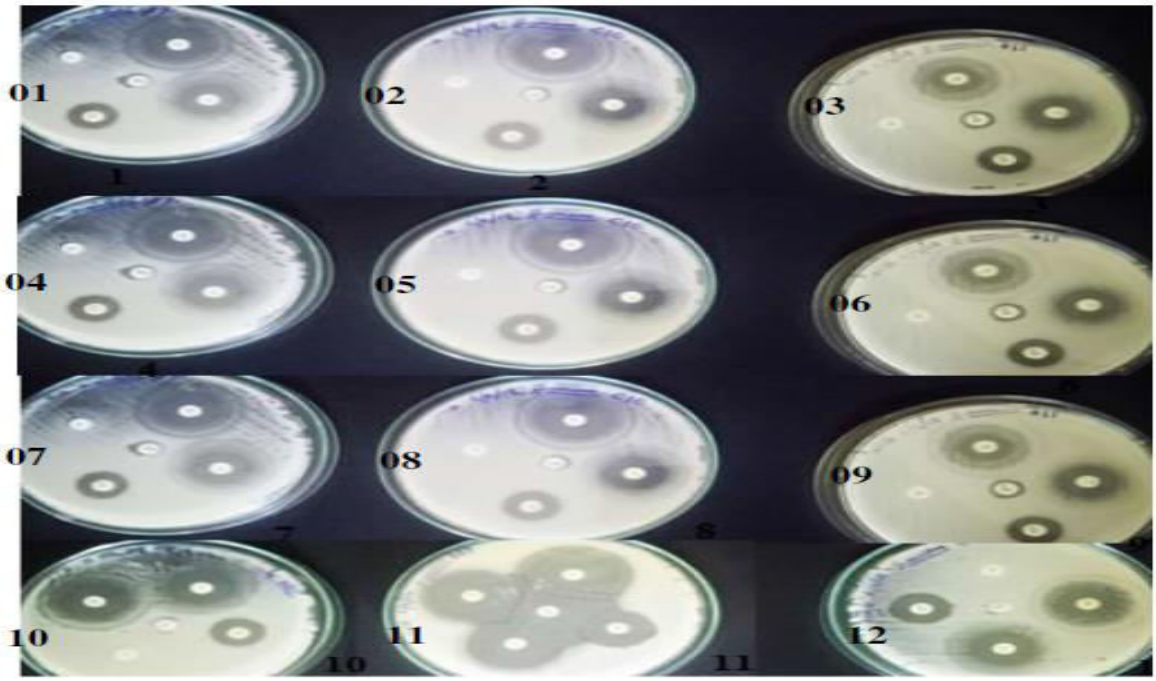

Fig. 2: Antibiotic assay for confirmation of the activity of penicillin, clindamycin, methicillin, vancomycin and lincomycin against *S. aureus* medical isolates.

Table 1: Antibiogram assay of *S. aureus* using five different antibiotics commonly used against *S. aureus* [4]

Sl. No.	Isolate No.	Penicillin 10mcg (12-17)	Clindamycin 2mcg (15-20)	Methicillin 5mcg (17-22)	Vancomycin 30mcg (14-17)	Lincomycin 10mcg (14-24)
01	001	<10	22	0	16	21
02	003	17	31	13	22	35
03	005	0	19	0	18	19
04	006	<10	22	0	15	19
05	008	<10	23	0	16	20
06	009	10	15	0	15	17
07	012	12	20	11	15	18
08	014	<10	21	0	14	18
09	015	<10	20	0	14	18
10	016	<10	15	0	14	17
11	019	10	0	0	0	0
12	020	0	13	0	15	19
MDR Activity		R-2,I-5,S-5	R-2, I-5, S-5	R-12	R-1, I-9, S-2	R-1,I-10,S-1

Legend: R-Resistant, I-Intermediate and S-Susceptible (based on CLSI chart, 2008)

Note: The resistance pattern of Antibiotic assay against each antibiotic (in % of total S. aureus screened)

Multi-drug resistance pattern: Penicillin: 83%; Clindamycin: 2%; Methicillin: 100%; Vancomycin: 8.3% and Lincomycin: 8.3%

3.3. Co-culture assay

Co-culture assay of fungal isolates against *S. aureus* isolates measured with 4 criteria, 4 distance inhibition, zone line, contact inhibition and over growth.

The co-culture assay activity of the fungal isolates tested against the multi-drug resistant isolates (Fig. 3) are as following;

1. Five showed contact inhibition (A01, A05, A07, A09 and A11),
2. Three showed overgrowth inhibition (A02, A06 and A10)
3. Two were showing distance inhibition (A04 and A08).
4. Two were (A03, A12) showing zone-line inhibition against *S. aureus* isolates

Fig. 3: Co-culture assay showing different interactions of *S. aureus* with fungal isolate (F3) during screening for secondary metabolites.

4. DISCUSSION

In the present study, we present the metabolite producing ability of fungi (F3), antagonizing the *S. aureus* activity. The fungi in normal functioning of their physiology and metabolism are known to produce few regular and common metabolites known for its own metabolic activity and function. Co-culture assay being a powerful tool to the progression of synthetic biology [12] and during our assay has shown to activate some of the probable gene clusters and their metabolic pathways leading to the production of novel secondary metabolites. The same has also been reported using *A. nidulans* capable of producing aspergicin and neoaspergillic acid being evaluated as potential antibiotics against the Gram-positive bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Bacillus dysenteriae*, *Bacillus proteus*and against the Gram-negative *Escherichia* coli [19] *S. aureus*, screened against five different commonly prescribed antibiotics showed resistant to penicillin and methicillin antibiotics, proving that 10 of the *S. aureus* isolates are penicillin resistant (83%) but it was all 12 isolates being resistant to methicillin (100%), being MRSA (Tab.1). The isolated fungi (F3) during co-culture assay produced some functional metabolites that are efficient against some of the multi-drug resistant *S. aureus* as indicative in the plates of the co-culture assay (Fig. 3). Hence we derive here that the co-culture assay is one of the efficient tool to evaluate the enhancement/ activation in metabolite production ability and probable activation of the functional gene clusters that has also been previously reported [15, 5] by co-culture assay.

5. CONCLUSION

Thereby we derive by our experimental evidence that using a powerful tool co-culture assay that can enhance, diverse anti-bacterial metabolite production ability of the fungal isolates against the multi-drug resistant *S. aureus*. It will be the best solution for the medical issues to provide proper drug by such organisms and which is safer and effective in microbial infection.

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