Antibacterial Dynamics Of Mucor Species Against Multi-Drug Resistant Bacterial Species

Sidra Noureen, Muhammad Shahzad, Ayesha Naeem, Azka Rizvi, Zunira Mughis

Abstract: These Emergence of antibiotic resistance in pathogens poses difficulties in treatment of bacterial infection. So discovery and development of alternative antibiotics is a need of hour. Present study was designed to screen Mucor species for antibacterial activity against multi-drug resistance bacterial pathogens. Mucor isolated from animal rations (n=30) were screened for toxin production by thin layer chromatography (TLC). Non-toxigenic isolates were confirmed by polymerase chain reaction (PCR). The cell free supernatants (CFS) of isolates (n=10) were evaluated against characterized multi-drug resistant bacteria; Staphylococcus aureus, Bacillus cereus, pseudomonas aeruginosa and Escherichia coli. Seven days old cell free supernatants of Mucor species was evaluated for antibacterial activity by agar well diffusion and minimum inhibitory concentration (MIC) was determined by micro-broth dilution method. One among nine non-toxigenic Mucor species revealed antibacterial activity against MDR-bacteria with mean zone of inhibitions 8.00± 1.00, 12.00± 1.00, 7.00± 1.00 and 11.00± 1.00 for MDR Escherichia coli, S.aureus, B.cerus and P.aeruginosa respectively. The lowest MIC (6.25µL / mL) determined against MDR-S.aureus followed by 12.5 µL / mL for MDR Escherichia coli, Bcerus and P.aeruginosa. Cytotoxicity evalution on Vero cell line indicated that Mucor e ≤ 6.25µL / mL is a safe concentration. It is concluded that Mucor specie have antibacterial potential against MDR-bacterial isolates.

Keywords: Antibacterial potential, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Mucor spp.

1 INTRODUCTION

Fungal species are predominant producers of bioactive compounds contributing 42 per cent among natural sources of these compounds. Aspergillus and Penicillium are major producers having potential production for wide variety of enzymes, drugs and antimicrobials [1]. Antibiotics are antimicrobials having ability to kill or inhibit bacterial growth. Several antibiotics are produced by fungi [2]. The first antibiotic Penicillin was obtained from Penicillium chrysogenum and successfully used to treat a bacterial infection [3]. Cephalosporins obtained from Acremonium are active against a wide variety of gram's positive and gram's negative bacteria [4]. Recently, some novel antibiotics have been discovered from fungal sources. Plectasin obtained from Actinomycetes pseudoplectania nigrella; a saprophytic fungus; has therapeutic potential against resistant strains of Staphylococcus aureus [5]. Copsin is also a novel product of fungi having antibacterial potential against Staphylococcus aureus, Bacillus subtilis and wide range of other gram's positive bacteria (Essig et al., 2014). Marine fungi are a golden source of novel bioactive metabolites [6]. Endophytic fungi are also known for the production of active novel metabolites [7]. Over 1200 endophytic fungi are reported for novel antibiotic production [8]. Infectious diseases have remained a major cause of mortality in past. However, antibiotic discovery markedly helped to conquered infectious diseases [9]. Excessive use or misuse of antibiotics led to resistance development in pathogens ultimately causes therapeutic problems [10, 11]. Now a day, antibiotic resistance have become a global challenge and microorganisms are developing and disseminating resistance through genetic element in hospitals and environmental isolates. These result in emergence of multi-drug resistance (MDR) and pan-drug resistance (PDR) in pathogens [12]. The term multidrug resistance (MDR) describes about resistance in microorganisms to three or more than three classes of antibiotics in in-vitro susceptibility testing [13]. Multi-drug resistant bacteria are increasing in health care as well as in community and difficult to treat with existing antibiotics. Bacterial isolates commonly show multi-drug resistance are Staphylococcus aureus, Klebsiella pnumoniae, Pseudomonas aeruginosa, Escherichia coli, Acinetobacter baumanii [12].

Emerging resistance in bacterial pathogens has emphasized not only on alternative therapeutic options but search for new antibiotics to curtail MDR and PDR pathogens. Present study was conducted on evaluation of antibacterial potential of Mucor species against multidrug resistant bacterial isolates of Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Bacillus cereus.

2 MATERIAL AND METHOD

Mucor species isolated from animal rations were assessed for antibacterial potential against MDR isolated of E. coli, Staphylococcus aureus, Pseudomonas aeruginosa and Bacillus cereus.

2.1 Stage Isolation and Identification of Mucor Species

Animal rations (n=30) were collected from local market and inoculated on Sabouraud's dextrose agar plates followed by incubation at 25±3 ^{0}C for 24 h. Isolates were purified and identified on the base of macroscopic and microscopic characters [14].

2.2 Screening for non-toxigenic isolates

Isolates of Mucor species were screened for mycotoxin production by thin layer chromatography (TLC) as described in [15]. Non-toxigenic isolates were selected for further experiments.

2.3 Extraction of metabolite from Mucor spp.

Mucor species (n=10) were grown in 100 mL Sabouraud's

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dextrose broth (SDB) by inoculating standard spore suspension (10^6 spores) in separate flask under sterilized conditions. Following the incubation at 25 °C for 7 days it was centrifuged at 3000 rpm for 30 min. Fungal mycelia setteled down in tube and cell free supernatent was filtred through 0.25µm membrane filter in [15].

2.4 Molecular characterization of isolates of Mucor spp.

Mucor species that showed antibacterial activity was confirmed by Polymerase chain reaction (PCR). Fungus was cultured in SDB and genomic DNA was extracted from pellet by kit method. PCR was carried out using specie specific primers. Mucor genus specific primers were used given as MucL1 as forward primer and MR1 as reverse primer:

MucL1 5' TGATCTACGTGACATATTTCT 3'

MR1: 5' AGTAGTTTGTCTTCGGTCAA 3.

A reaction mixture of 25 µL was prepared containing 12.5 master mixes, 2 µL of template DNA, 1.5 µL of each primer and 7.5 µL of distilled water. PCR was performed in thermo-cycler as follows; Initial denaturation was carried out for 1 cycle at 94 °C for 1 minute, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min. One cycle of final extension at 72 °C for 5 min was carried out. Agarose gel electrophoresis (1%) was carried out to analyse amplicons. Sample bands were compared with bands of ladder, run along sample as described in [16]. Observation of gel was carried out under Ultra violet (UV) at 260 nm.

2.5 Selection of Multi-Drug Rresistant Bacterial Isolates

Indigenously isolated Confirme Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Bacillus cereus were evaluated for antibiotic susceptibility assay by Kirby Bauer disk diffusion method to find out multidrug resistance (Balouiri et al., 2016). Following antibiotics were used; Amoxicillin (30 µg), Gentamicin (10 µg), Ampicillin (25 µg), Oxacillin (10 µg), Cefoxitin (30 µg), Levofloxacin (5 µg), Vancomycin (5 µg), Ceftriaxone (30 µg), Tylosin (15 µg), Erythromycin (15 µg), Bacitracin (15 µg) and Oxytetracycline (30 µg). Standardized bacterial inoculum equivalent to 0.5 McFarland was spread on Muller Hinton agar followed by application of discs. Plates were incubated for 24 h at 37 °C. Zones of inhibition (ZOI); if present; were observed and measured in millimetres (mm) and compared with the standards of Clinical Laboratory Standard Institute CLSI.

2.6 In vitro activity of metabolites extracted from Mucor spp.

Antibacterial activity of cell free supernatant of Mucor species was determined by agar well diffusion method as described in [17]. A standard inoculum of each fresh MDR bacterium equal to 0.5 McFarland was swabbed on Muller Hinton agar plates. Supernatant (50 uL) was poured into sealed wells in previously inoculated nutrient agar followed by incubation at 37 °C for 24 h. Zones of inhibition showing antibacterial activity were measured in millimetre (mm).

2.7 Determination of Minimum Inhibitory Concentration

The lowest concentration of antibacterial metabolite which can inhibit visible growth of bacteria after overnight incubation was calculated by broth micro-dilution method as followed in [18]. Two fold serial dilutions of cell free supernatant were prepared in micro-titration plates. Standard MDR bacterial culture was added in each well except last well. Optical density (OD) was measures and recorded by ELISA reader at 595 nm wavelength and MIC values were calculated.

2.8 Cytotoxicity profile

Cytotoxicity of CFS showing antibacterial activity was checked MTT (by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay [19]. Monolayer of Vero cells was developed in cell culture media (M-199) in 96 well plates. Two-fold serial dilution of cell free supernatant was prepared and 100 µl of each dilution was added in wells having monolayer of cells. Followed by incubation at 37 °C for 24 h 100 uL MTT dye (5 mg/mL) was added and incubated for 3 h. Optical density was measured at 570 nm cell survival percentage was calculated.

3 RESULTS

Isolates of Mucor (n= 10) isolated from animal ration were confirmed by polymerase chain reaction (PCR) and evaluated for antibacterial activity against MDR bacterial isolates.

3.1 Isolation and Identification of Mucor

SDA. Growth of each isolate was observed by macroscopic and microscopic methods. The colonies were initially white and fluffy turning to brownish on obverse side and white on reverse side of plate. Microscopy revealed coenocytic hyaline hyphae, presence of sporangium and sporangiophores.

3.2 Selection of non-toxigenic Mucor species

Screening for non-toxigenic fungi was carried out by TLC. Ten Mucor isolates were tested for mycotoxin production. Out of ten, one isolate (10%) was positive for mycotoxin production and 9 (90%) isolates were non-toxigenic (Fig. 1).

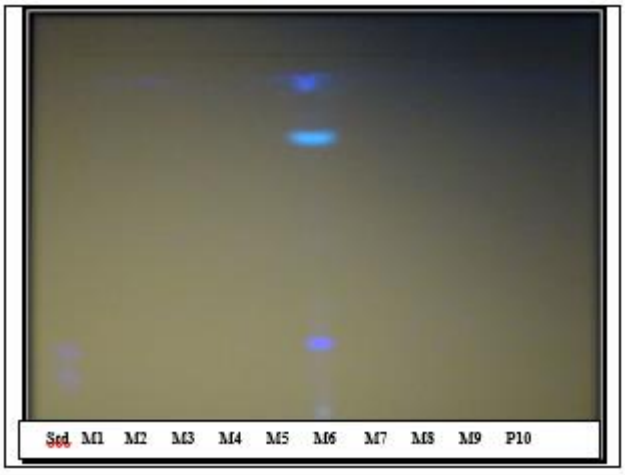

***Fig. 1**. Chromatogram (TLC) of Mucor spies (n=10) under UV-light. Fluorescence indicates the presence of mycotoxins in sample*

3.3 Molecular Characterization of Mucor Species

Mucor species was confirmed by PCR. Isolates produced an amplicon of 830 bps were considering Mucor (Fig. 2). In Figure after ladder there is a positive control followed by band of representative Mucor species.

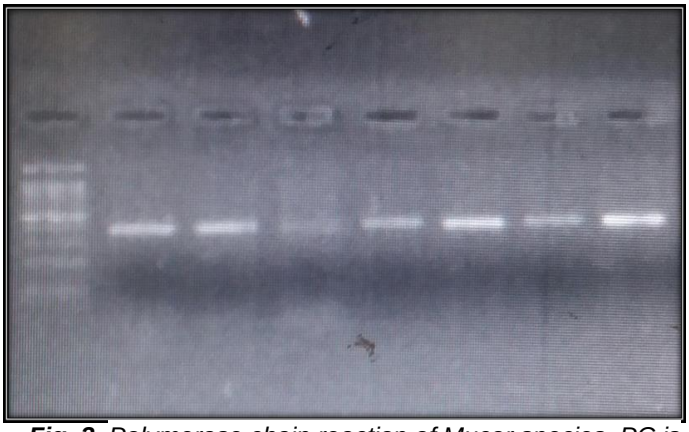

***Fig. 2**. Polymerase chain reaction of Mucor species. PC is positive control*

3.4 Selection of Multi-drug resistant bacterial isolates

Isolates of Escherichia coli, Staphylococcus aureus, Bacillus cereus and Pseudomonas aeruginosa were evaluated for antibiotic resistance by disc diffusion method against 12 antibiotics. Escherichia coli showed highest resistance (100%) to amoxicillin, oxytetracyclin, bacitracin erythromycin, tylosin, vancomycin, ampicillin, levofloxacin, cefoxitin and oxacillin. Escherichia coli appeared as sensitive only against gentamicin and ceftriaxone. Staphylococcus aureus appeared were completely (100%) resistant to amoxicillin, bacitracin erythromycin, ceftriaxone tylosin, vancomycin, ampicillin, cefoxitin and oxacillin. Staphylococcus aureus were found sensitive to levofloxacin, gentamicin and oxytetracyclin. Bacillus cereus was resistant to amoxicillin, bacitracin, gentamicin, erythromycin, tylosin, vancomycin, ceftriaxone, ampicillin, cefoxitin and oxacillin and sensitive to levofloxacin and oxytetracyclin. Pseudomonas aeruginosa were resistant to all tested antibiotics i.e. Amoxicillin, bacitracin erythromycin, ceftriaxone tylosin, vancomycin, ampicillin, cefoxitin, oxacillin, levofloxacin, gentamicin and oxytetracyclin. Escherichia coli, Staphylococcus aureus, Bacillus cereus and Pseudomonas aeruginosa appeared as MDR.

3.5 Antibacterial activity

Mucor species (n=9) appeared as non-toxigenic were tested for antibacterial activity by well diffusion and microbroth-dilution method against multiple drug resistant isolates of Escherichia coli, Staphylococcus aureus, Bacillus cereus and Pseudomonas aeruginosa. Zone of inhibition showing antibacterial activity were measured in millimeter (mm). Among nine non-toxigenic isolates one isolate of Mucor showed antibacterial activity against Staphylococcus aureus, B. cereus, E. coli and Pseudomonas aeruginosa. The highest zone of inhibition observed was 12mm against Staphylococcus aureus. The lowest zone of inhibition given recorded was 7 mm against B. cereus (Table 1). Minimum inhibitory cell free supernatant of Mucor specie concentration of cell free supernatant of Mucor determined was 12.5 µL/mL against E. coli, 6.25 µL/mL against Staphylococcus aureus, 12.5 µL/mL against B. cereus and 12.5 µL/mL against Pseudomonas aeruginosa (Fig. 3).

***Table 1** Antifungal activity of cell free supernatant of Mucor species against MDR bacteria*

Mucor isolate	Zone of inhibition (mm)			
	E.coli	S.aureus	B.cereus	P.aeruginosa
M1	00±00[a]	00±00[a]	00±00[a]	00±00[a]
M2	00±00[a]	00±00[a]	00±00[a]	00±00[a]
M3	8.00± 1.00[c]	12.00±1.00[d]	7.00± 1.00[b]	11.00±1.00[d]
M4	00±00[a]	00±00[a]	00±00[a]	00±00[a]
M5	00±00[a]	00±00[a]	00±00[a]	00±00[a]
M6	00±00[a]	00±00[a]	00±00[a]	00±00[a]
M7	00±00[a]	00±00[a]	00±00[a]	00±00[a]
M8	00±00[a]	00±00[a]	00±00[a]	00±00[a]
M9	00±00[a]	00±00[a]	00±00[a]	00±00[a]

3.6 Evaluation of cytotoxic effect of antibacterial metabolite

Cytotoxicity of metabolite of Mucor isolates was checked by MTT assay and cell survival percentage of Vero cells was calculated (Table 2). Safe concentration of cell free supernatant of Mucor appeared 6.25 µL/mL and concentration below 6.25 µL/mL.

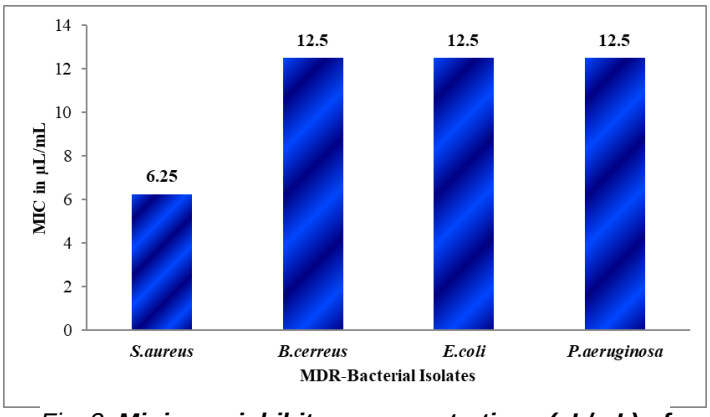

*Fig. 3**. Minimum inhibitory concentrations (µL/mL) of***

***Table 2 Cell** survival percentage of fungal isolates producing antibacterial metabolites*

	1	2	3	4	5	6	7	8	9	10
Concentration µL/mL	100	50	25	12.5	6.25	3.125	1.562	0.781	0.39	0.19
Percentage survival	0	4	15.29	38	53	63	74	76	80	83

4 Discussion

Bacteria are ubiquitous in nature and contaminate food, pharmaceutical and cosmetic products. The bacterial transmission to human beings results in disease production. Several antibacterial agents have been used to treat bacterial infections. Now a day bacterium are getting resistant to available antibacterial agents. So there is need to discover alternative compounds to treat infections caused by MDR

bacteria. One of the alternatives is antimicrobials produced by fungi [20]. Mycotoxins are hazardous thermo-stable fungal metabolites may cause poisoning termed as mycotoxicosis in human and animals. Mycotoxin production may interfere with purification of beneficial products from fungi. So it is preferred to select non-toxigenic isolates for cost-effective production on industrial level [21]. Thin layer chromatography is the cheapest method for screening of non-toxigenic and toxigenic fungi [22]. In current study non-toxigenic Mucor species were selected for antibacterial activity against MDR bacteria. Filamentous fungi isolated from river sediment were evaluated for antibacterial activity against MDR bacteria. Most of the species belong to genus Aspergillus. The cell free supernatants elaborated bactericidal activity against Staphylococcus aureus, Methicillin resistant Staphylococcus aureus, Enterococcus fecali, E. coli and Klebsiella pnumonae [23]. Study on antibacterial of endophytic fungi isolated from leaves of Indigofera suffruticosa Miller revealed inhibitory effect against Grams positive and Grams negative bacteria. According to this study best results were against S.aureus with 1.56mg/mL MIC [24]. Leohumicola is one of the mycorizal fungi have been found as antimicrobial against Gram positive (Bacillus subtilis and Staphylococcus aureus) and Gram negative bacteria (Escherichia coli, Serratia marcescens, Proteus vulgaris, Shigella sonnei and Klebsiella pneumonia). The antibacterial activity was recorded against B. subtilis, Staphylococcus aureus and Proteus vulgaris [25]. Mushroom is an edible and medicinal fungus was found to be antibacterial against Staphylococcus aureus and Pseudomonas aeruginosa. These mashrooms were Amanita virosa and Cortinarius praestans. Similarly antimicrobial activity was determined in endophytic fungi Trucatella hartigii against E. fecalis and S. aureus [26]. Mucor ruxii was evaluated for antibacterial activity by the production of chitosan against E. coli and Micrococcus leutus. It was concluded that Mucor ruxii produced biocidal compound and inhibit the growth of E. coli and Micrococcus leutus [27]. Above mentioned studies strengthen the present study. Several fungi from different sources produce bioactive compounds which possess antibacterial activity even against multi-drug resistant bacteria.

5 Conclusion

Development of antibiotic resistance is a global issue. Pathogens recognized as MDR cannot be treated by available antibiotics. So, there is a need to discover and develop new antimicrobials. Fungi are the best option due to diverse metabolism. In current study, Mucor specie is found effective to inhibit MDR Escherichia coli, Staphylococcus aureus, Bacillus cereus and Pseudomonas aeruginosa. However, there is need of further experimentation on characterization of these metabolites.

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Disclosure

“The authors declare that there is no conflict of interests regarding the publication of this article”.

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