Molecular basis of resistance to echinocandins in clinical Candida glabrata isolates in Kuwait

*Al-Baghsami Z, Ahmad S, Khan Z
Department of Microbiology, Faculty of Medicine, Kuwait University, Kuwait

Introduction

1. Candida species are normal microbiota inhabiting several sites in the body, oral cavity, vaginal and gastrointestinal tracts. Candida species are the most common cause of invasive fungal infections (IFI) which are associated with high morbidity and mortality.
2. Candida albicans ranks the top causative agent of IFI. But infections due to non-Candida albicans-Candida species have increased significantly in the past two decades, due to opportunistic pathogens like Candida glabrata.
3. Candida glabrata is the second/third most common Candida species causing candidemia or invasive candidiasis in high risk patients. Significantly higher prevalence of C. glabrata is seen in immunocompromised patients and patients with previous exposure to antifungal drugs especially azoles.
4. Echinocandin antifungal drugs includes caspofungin, micafungin, and anidulafungin and are the first-line therapy of treatment of candidemia. They inhibit β-(1,3)-D-glucan synthase which is involved in cell wall biosynthesis and is encoded by three FKS genes. Resistance to echinocandins has emerged in C. glabrata due to prolonged drug exposure and is increasing.
5. Amino acid substitutions in hot spot 1 (HS-1) and hot spot 2 (HS-2) of FKS1 and FKS2 genes are associated with echinocandin resistance. The most common amino acid substitutions were noted in HS-1 (like 562P) of FKS1, and HS-1 (like 563P) of FKS2.

Methods

1. A total of 75 C. glabrata isolates were studied. The isolates were initially identified phenotypically by Vitek2 system.
2. Identity of the isolates were confirmed by multiplex PCR assay (mPCR) by using species specific primers that simultaneously detect C. glabrata, C. nivariensis and C. bracarensis.
3. Molecular identification was also confirmed by sequencing the internal transcribed spacer (ITS) region & BLAST search was used for species identification for selected isolates.
4. Antifungal susceptibility testing to micafungin was determined by E-test. MICs were read after 24hrs and EUCAST breakpoints were followed: MICs < 0.032μg/mL and MICs > 0.032μg/mL, resistant.
5. The HS-1 and HS-2 of both FKS1 and FKS2 were amplified by PCR from 75 C. glabrata isolates and both strands of purified amplicons were sequenced with internal primers (Table 1). Mutations were detected by sequence comparisons with reference strain by using Clustal Omega.
6. Molecular relatedness among resistant isolates was determined from concatenated sequence data from ITS/mPCR1/FKS1/FKS2 by MEGA software.

Results

1. The 75 C. glabrata isolates were obtained from different clinical specimens which included blood (n=12), urinal (n=27), BAL and sputum (n=11), skin swab (n=6), vaginal swab (n=3) and other specimens (n=16) (Table 2).
2. Multiplex PCR with DNA from reference strains was done by using species-specific primers corresponding to C. glabrata, C. nivariensis and C. bracarensis yielded an amplicon of 212 bp from C. glabrata, 299 bp from C. bracarensis and 411 bp from C. nivariensis (Fig 1).
3. All 75 isolates were identified as C. glabrata by Vitek2 and also by mPCR as they yielded an amplicon of 212 bp, which is characteristic of C. glabrata.
4. Based on EUCAST breakpoints, 71 (95%) of 75 isolates were susceptible showing MICs <0.032μg/mL whereas 4 (5%) isolates were resistant to micafungin (MYC) with MICs >0.032μg/mL. The MICs for amphotericin B (AMP), fluconazole (FLU) and caspofungin (CAS) were collected along with (MYC) for comparison purpose (Table 3).
5. None of the 71 phenotypically susceptible isolates contained a non-synonymous mutation in HS-1 or HS-2 of both FKS1 and FKS2. Four isolates contained synonymous mutation at codon position 625 of FKS1 gene.
6. The HS-1 and HS-2 of both FKS1 and FKS2 were amplified from all 75 C. glabrata isolates (Fig 3). The amplicons were purified and sequenced with internal primers. DNA sequence data showed that three of four isolates phenotypically resistant to micafungin contained a non-synonymous mutation (566C) in HS-1 of FKS2 gene while another isolate carried a deletion of 3 nucleotides at codon 658 resulting in deletion of Pho658 which is next to HS-1 in the same gene (Table 4).
7. All four echinocandin-resistant C. glabrata isolates were found to be genotypically distinct strains based on comparisons of concatenated sequences of ITS region together with HS-1 and HS-2 of FKS1 and FKS2 gene (Fig. 4).

Table 1: List of the primers with their nucleotide sequences that were used for PCR amplification of HS-1 and HS-2 of FKS1/FKS2 and sequencing of amplicons

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Direction</th>
<th>Target Gene</th>
<th>Target region</th>
<th>Nucleotide sequence</th>
<th>Purpose</th>
<th>Product size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGFKS1-F2</td>
<td>forward</td>
<td>FKS1/FKS2</td>
<td>HS1</td>
<td>5'-ATGCCATGNaTGCATTCTCGAC-3'</td>
<td>PCR</td>
<td>560</td>
</tr>
<tr>
<td>CGFKS1-R1</td>
<td>reverse</td>
<td>FKS1</td>
<td>HS1</td>
<td>3'-GGGTCTCCATAGTAACCCCGA-5'</td>
<td>sequencing</td>
<td>N. A.</td>
</tr>
<tr>
<td>CGFKS1-F2S</td>
<td>forward</td>
<td>FKS1</td>
<td>HS3</td>
<td>5'-GAAGGCTACACAGCCATCAGTAC-3'</td>
<td>sequencing</td>
<td>N. A.</td>
</tr>
<tr>
<td>CGFKS1-R1S</td>
<td>reverse</td>
<td>FKS1</td>
<td>HS3</td>
<td>3'-GGCGGCTTCAAGGGAAGATGTC-5'</td>
<td>sequencing</td>
<td>N. A.</td>
</tr>
<tr>
<td>CGFKS2-F2</td>
<td>forward</td>
<td>FKS1/FKS2</td>
<td>HS2</td>
<td>5'-GTTGAAACATTGTTCTGGCGAC-3'</td>
<td>PCR</td>
<td>538</td>
</tr>
<tr>
<td>CGFKS2-R1</td>
<td>reverse</td>
<td>FKS1/FKS2</td>
<td>HS2</td>
<td>3'-GACCAACTTGAGTAAACAGTAGA-5'</td>
<td>sequencing</td>
<td>N. A.</td>
</tr>
<tr>
<td>CGFKS2-F2S</td>
<td>forward</td>
<td>FKS1/FKS2</td>
<td>HS3</td>
<td>5'-TGTCATCAATGAGGACGACGA-3'</td>
<td>sequencing</td>
<td>N. A.</td>
</tr>
</tbody>
</table>

N.A. not applicable

Table 2: Distribution of C. glabrata in different clinical specimens and the percentage of isolates susceptible or resistant to micafungin

<table>
<thead>
<tr>
<th>Period</th>
<th>No. of isolates</th>
<th>Minimum inhibitory concentration (MIC)</th>
<th>Susceptible (%)</th>
<th>Resistant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008-2017</td>
<td>Blood</td>
<td>12</td>
<td>0.032</td>
<td>0</td>
</tr>
<tr>
<td>2008-2017</td>
<td>Sputum</td>
<td>8</td>
<td>0.032</td>
<td>0</td>
</tr>
<tr>
<td>2008-2017</td>
<td>Vaginal swab</td>
<td>3</td>
<td>0.032</td>
<td>0</td>
</tr>
<tr>
<td>2014-2016</td>
<td>Urine</td>
<td>6</td>
<td>0.032</td>
<td>0</td>
</tr>
<tr>
<td>2014-2016</td>
<td>BAL</td>
<td>3</td>
<td>0.032</td>
<td>0</td>
</tr>
<tr>
<td>Total 2017</td>
<td>Others</td>
<td>16</td>
<td>0.032</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3: Antifungal susceptibility profile of 7 selected C. glabrata isolates

<table>
<thead>
<tr>
<th>sl. no.</th>
<th>Isolate</th>
<th>Clinical source</th>
<th>Minimum inhibitory concentration (MIC)</th>
<th>mPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2487/06</td>
<td>Vag. Swab</td>
<td>0.032</td>
<td>0.032</td>
</tr>
<tr>
<td>2</td>
<td>105/13</td>
<td>ET aspirate</td>
<td>0.032</td>
<td>0.032</td>
</tr>
<tr>
<td>3</td>
<td>567/14</td>
<td>Urine</td>
<td>0.032</td>
<td>0.032</td>
</tr>
<tr>
<td>4</td>
<td>164/15</td>
<td>Urine</td>
<td>0.032</td>
<td>0.032</td>
</tr>
<tr>
<td>5</td>
<td>458/16</td>
<td>Urine</td>
<td>0.032</td>
<td>0.032</td>
</tr>
<tr>
<td>6</td>
<td>355/16</td>
<td>Urine</td>
<td>0.032</td>
<td>0.032</td>
</tr>
<tr>
<td>7</td>
<td>2138/17</td>
<td>Urine</td>
<td>0.032</td>
<td>0.032</td>
</tr>
</tbody>
</table>

Table 4: FKS gene sequence results in 7 selected C. glabrata. Mutations in hot spot (HS-1) of FKS2 are marked in bold

<table>
<thead>
<tr>
<th>sl. no.</th>
<th>Clinical source</th>
<th>Gene and isolate region</th>
<th>FKS1</th>
<th>FKS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vag. Swab</td>
<td>HS-1</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>2</td>
<td>ET aspirate</td>
<td>HS-1</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>3</td>
<td>Urine</td>
<td>HS-1</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>4</td>
<td>ET aspirate</td>
<td>HS-2</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>5</td>
<td>Urine</td>
<td>HS-2</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>6</td>
<td>Urine</td>
<td>HS-2</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>7</td>
<td>Urine</td>
<td>HS-2</td>
<td>wt</td>
<td>wt</td>
</tr>
</tbody>
</table>

Conclusions

1. Although echinocandin resistance among Candida species is rare, its incidence in C. glabrata is increasing resulting in clinical failure among infected patients.
2. Four of 75 (5.3%) C. glabrata isolates were resistant to micafungin.
3. Our data also show that all 4 echinocandin-resistant C. glabrata isolates in Kuwait contained mutations in HS-1 of FKS2 which are associated with high-level resistance to micafungin and other echinocandins.
4. Rapid detection of FKS mutations will contribute to rapid diagnosis of echinocandin-resistant C. glabrata infection and will help in better management of infected patients.

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A comparison of Multiplex PCR, ERIC-PCR, 16S rDNA sequencing, and Whole Genome sequencing for identification and/or typing of clinical Acinetobacter baumannii strains isolated in two major hospitals in Kuwait

*Nasser K1, Khan MW2, Purohit P3, Al-Obaid I3, Dhar R4, Al-Fouzan W4, Mustafa AS1

1Department of Microbiology, 2OMICS Research Unit, Research Core Facility, Faculty of Medicine, Kuwait University. 3Department of Medical Microbiology, Al-Sabah Hospital, Kuwait. 4Department of Medical Microbiology, Farwaniya Hospital, Kuwait

Introduction

Acinetobacter baumannii is a rising concern in the medical field as one of the most dangerous MDR (multiple drug resistant) opportunistic bacterial pathogens (ESCAPE pathogens) in nosocomial settings [1, 2]. The phenotypic methods for identification and typing of A. baumannii are unsatisfactory in distinguishing between A. baumannii and genotypically related species of the Acinetobacter baumannii-calcoaceticus complex [3].

Materials and Methods

1. Sixty-seven phenotypically identified clinical strains of A. baumannii, isolated at Al-Sabah, and Farwaniya hospitals in Kuwait, were used in this study.
2. Qiagen kits were used to isolate the genomic DNA from the bacterial cultures, after which the isolated DNA were quantified by Qubit fluorometry to be used for multiplex PCR, ERIC-PCR, 16S rDNA sequencing, and Whole Genome Sequencing using standard methods.
3. Multiplex PCR was performed with 4 primer pairs (P-rA1/2, Sp4F/R, P-Ab-ITSF/R, P-AGS3-F/R), and the PCR products were run on traditional agarose gels.
4. 16S rDNA sequencing was performed using MicroSeq 500 16S rDNA kits from Applied Biosystems.
5. ERIC-PCR product was run on a Bioanalyzer DNA7500 chip from Agilent to obtain banding patterns.
6. Whole Genome Sequencing was performed according to the instructions provided by Illumina for MiSeq system Nextera kits.
7. The band profiles (in case of multiplex PCR and ERIC-PCR) and the DNA sequence data (in case of 16S rDNA and Whole Genome Sequencing) were analyzed by BioNumerics software for species identification and genotyping analysis.
8. Discriminatory indices were calculated using the online tool http://insilico.ehu.es/mini_tools/discriminator_y_power/index.php

Results

1. All of the 67 isolates were identified as A. baumannii by multiplex PCR (Fig 1) and 16S rDNA sequencing (Fig 2).
2. The clustering patterns of 16S rDNA (Fig 2), ERIC-PCR (Fig 3), and Whole genome Sequencing single nucleotide polymorphisms (SNP)-based analyses gave a discriminatory index of 0.37, 0.52, 0.36, respectively (Table 1).
3. Furthermore, ERIC-PCR divided the isolates in maximum number of groups and isolates per group with the highest discriminatory index, at a value of 0.52 (Table 1).

Conclusions

1. The molecular methods used in this study confirmed the identity of the isolates as A. baumannii and suggest genetic heterogeneity among them.
2. However, the ERIC-PCR showed the maximum groups/genotypes with highest discriminatory power.

References

Introduction

The increasing incidence of multidrug-resistant tuberculosis (MDR-TB, i.e. resistant to rifampicin and isoniazid) is now considered one of the most serious public health challenges in the world. The disease is caused by Mycobacterium tuberculosis, a Gram-positive bacterium that infects the lungs and other organs, primarily the liver. M. tuberculosis is responsible for the vast majority of cases of TB, with over 10 million cases diagnosed each year. The World Health Organization (WHO) estimates that there are around 1.4 million new cases of drug-resistant tuberculosis each year, which is a significant proportion of the total cases worldwide.

Methods

1. The study was conducted using a combination of molecular and epidemiological methods. The patients were interviewed and their medical records reviewed to obtain data on their demographic characteristics, clinical history, and drug resistance.
2. The isolates were identified and typed using the spoligotyping method, which provides information on the genetic diversity of the M. tuberculosis strains. The isolates were also analyzed using the RD1 target region, which is highly polymorphic and useful for the identification of specific clones.
3. The drug susceptibility testing was performed using the proportionate method of the Bactec MGIT system.
4. The isolates were examined for the presence of mutations in the katG and inhA genes, which are associated with resistance to isoniazid and rifampicin, respectively.
5. The MDR-TB patients were grouped based on the number of drug-resistant strains detected.
6. The isolates were also analyzed for the presence of mutations in the embB gene, which is involved in the synthesis of the drug ethambutol.

Results

1. All isolates were identified as M. tuberculosis using the spoligotyping method. The majority of the isolates were from Indian expatriates.
2. The isolates were also analyzed for the presence of mutations in the katG and inhA genes, which are associated with resistance to isoniazid and rifampicin, respectively.
3. The drug resistance patterns were analyzed using the Bactec MGIT system.

Conclusions

1. The results of the study indicate that the incidence of MDR-TB is higher in expatriate individuals, particularly in the Indian population. This highlights the need for increased public health awareness and targeted interventions to prevent the spread of drug-resistant TB.
2. The study also emphasizes the importance of routine drug susceptibility testing and the need for improved diagnostic tools to accurately identify drug-resistant TB cases.
3. The results suggest that targeted interventions, such as the provision of isoniazid prophylaxis to contacts of MDR-TB cases, could be effective in reducing the transmission of drug-resistant strains.

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