Susceptibilities of Malassezia strains from pityriasis versicolor, Malassezia folliculitis and seborrheic dermatitis to antifungal drugs

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ABSTRACT

The human pathogenic yeast genus Malassezia may be an etiological agent of skin disorders and has received considerable attention from dermatologists in recent years. To investigate the different susceptibilities of Malassezia species to four antifungal drugs, we isolated a total of 244 Malassezia strains and identified six species of Malassezia from patients with clinical skin diseases. The minimum inhibitory concentration (MIC) of the four antifungal drugs was obtained by comparing the susceptibility of the isolated Malassezia strains to four antifungal drugs (ketoconazole (KTZ), itraconazole (ITZ), fluconazole (FLC) and amphotericin B (Am B)). We demonstrated that M. furfur, M. sympodialis, M. pachydermatis and M. globosa are the most common Malassezia species in the three skin diseases. The MICs of KTZ, ITZ, FLC and Am B against M. furfur, M. sympodialis, M. pachydermatis and M. globosa ranged from 0.03 - 16 mg/L, 0.03 - 2.0 mg/L, 0.03 - 8 mg/L, and 13 - 64 mg/L, respectively. The sensitivities of Malassezia to the four antifungal drugs from high to low were ITZ > KTZ > Am B > FLC. The susceptibilities of the various Malassezia species to the four antifungal drugs were different, and the susceptibility of M. furfur to KTZ was significantly different from those of the three skin diseases (pityriasis versicolor, Malassezia folliculitis and seborrheic dermatitis). Our results suggested that the MIC analysis of the four antifungal drugs would be helpful in preventing drug resistance in the clinical screening of Malassezia and choosing better antifungal drugs to treat Malassezia-associated skin diseases.

1. Introduction

Malassezia, one of the resident bacteria on the human skin surface [1], is a lipophilic yeast-like fungus; Malassezia cultures require lipid supplementation for growth [2, 3]. Malassezia can directly infect the skin and cause seborrheic dermatitis, atopic dermatitis, psoriasis, hemorrhoids, onychomycosis, otitis externa and foreskin balanitis [4, 5]. In recent years, reports of systemic infections caused by Malassezia have gradually increased, and the skin infection caused by Malassezia manifests as many diseases, has a high incidence rate, recurs easy, and has a great impact on the physical and mental health of patients [6, 7, 8, 9, 10, 11, 12]. Commonly isolated Malassezia species have differences due to the different regions and countries they are isolated in and the populations they infect, and there are differences between healthy individuals and patients with various skin diseases [13].

There are many studies on the various Malassezia species causing diseases, such as pityriasis, Malassezia folliculitis and seborrheic dermatitis, and different species of Malassezia have been isolated from various regions [14, 15, 16]. Pityriasis and seborrheic dermatitis are mainly caused by M. furfur, followed by M. globosa, while Malassezia folliculitis is mainly caused by M. globosa; however, the distribution of the strains of these three diseases is different. In China, the highest

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detection rates of Malassezia in lesioned skin were observed for M. globosa, M. restricta, and M. sympodialis [17]. The dominant species of pityriasis include M. furfur in Indonesia [18] and M. globosa in Japan [19], while the dominant strains of pityriasis and seborrhoeic dermatitis are M. sympodialis and M. globosa in Canada [20] and M. furfur in Brazil [9]. Malassezia has been classified into over 14 species, and eight species of Malassezia have been isolated from human skin, including M. dermatis, M. furfur, M. globosa, M. japonica, M. obtusa, M. pachydermatis, M. restricta, M. sympodialis, M. slooffiae, and M. yamatoensis [21]. In the clinic, we have found that M. globosa, M. furfur and M. sympodialis are the dominant species of Malassezia infection in Kunming. Malassezia infection usually occurs in young and adult males. Pityriasis versicolor, Malassezia folliculitis, and seborrhoeic dermatitis are common skin diseases caused by Malassezia. Malassezia infection in Kunming accounts for 13.8% of superficial fungal infections and is the second highest fungal infection, which may be related to geographical environment and the consumption of spicy foods in this area. Therefore, the distribution and antifungal drug susceptibilities of Malassezia species in pityriasis versicolor, Malassezia folliculitis and seborrhoeic dermatitis need to be analyzed and will be important for guiding clinical treatment.

Although clinical studies have confirmed that fluconazole (FLC), itraconazole (ITZ) and ketoconazole (KZ) are effective in treating these skin diseases, the long-term safety of antifungal drugs remains unknown. The treatment cycle of fungal infections is generally long, and short-term medication cannot effectively cure the infection, while long-term medication not only is hazardous but also easily increases fungal resistance. In recent years, the systematic use of antifungal drugs, such as amphotericin B (Am B), to treat infections of sensitive Malassezia strains has been taken seriously. There are few studies on the susceptibility of Malassezia strains isolated from clinical samples to Am B [22, 23]. Álvarez-Pérez S conducted a drug susceptibility test on 60 Malassezia furfur strains and showed that the MIC value of Am B among those strains was <1 mg/L [23]. In this study, the drug susceptibilities of M. furfur, M. pachydermatis, M. sympodialis, and M. globosa to four antifungal drugs (KZ, ITZ, FLC and Am B) were analyzed, and the same species of Malassezia were isolated from different skin diseases.

2. Materials and methods

2.1. Malassezia strains and culture

A total of 244 Malassezia strains were isolated from patients with pityriasis versicolor, Malassezia folliculitis and seborrhoeic dermatitis, including 85 strains of M. globosa, 77 strains of M. furfur, 37 strains of M. sympodialis, 28 strains of M. pachydermatis, 10 strains of M. restricta, and 7 strains of M. slooffiae. Four standard strains of Malassezia (M. furfur CBS1878, M. sympodialis CBS7222, M. pachydermatis ATCC4791, and M. globosa CBS7990) were provided by the Medical Fungi Deposit Center of the Institute of Dermatology, Chinese Academy of Medical Sciences. This research was approved by the Ethics Committee of First Affiliated Hospital of Kunming Medical University. Written informed consent was obtained from all patients for the use of their samples and clinical records for our Malassezia analysis experiments.

Fatty acid RPMI 1640 medium was used to test the susceptibilities of eight Malassezia species to a new triazole, posaconazole, and to six established antifungal agents by a modified National Clinical Trial Committee for Standardization (NCCLS) M27-A2 microdilution method. Fatty acid RPMI 1640 medium was used to test the susceptibilities of six species of Malassezia to the four established antifungal drugs by a modified NCCLS (National Clinical Laboratory Standardization Institute) M27-A2 microdilution method [24]. The strains were cultured twice on Sabouraud’s medium containing olive oil at 32 °C for 5 days before the susceptibility test. After the second culture, 1-2 colonies with a diameter of ≥1 mm were collected; suspensions of the yeasts were made with 1 ml of sterile physiological saline and shaken for 15 s. Then, the concentrations of the yeast suspensions were diluted to 1 - 5 × 10^8 CFU/ml with Malassezia liquid medium. The final concentration of the yeasts was 0.5 - 2.5 × 10^6 CFU/ml.

2.2. Antifungal drugs

Itraconazole, fluconazole, and ketoconazole were purchased from TCI America (Portland, OR, USA), and amphotericin B was purchased from Vetech Laboratories (Guelph, Canada). Fluconazole was formulated into a 5,120 mg/L drug stock solution by sterilized distilled water; ketoconazole and itraconazole were first dissolved in dimethyl sulfoxide (DMSO), with DMSO concentrations less than 1%, and then diluted with sterile distilled water to prepare a 1,280 mg/L drug stock solution. The drug storage solution was sealed and stored at a temperature of -20 °C. The fluconazole stock solution was diluted 10 times with Malassezia liquid medium to a concentration of 128 mg/L, and this solution was twice the final concentration of the drug in the first well of the drug-sensitive plate (64 mg/L). The raw solutions of itraconazole and ketoconazole were diluted 50 times with Malassezia liquid medium to a concentration of 32 mg/L, which was twice the final concentrations of the two drugs in the first two wells of the drug-sensitive plate (16 mg/L).

2.3. Drug allergy testing

The in vitro susceptibility analysis of Malassezia was carried out according to the yeast microdilution method in NCCLS M27-A2. One hundred microliters of the concentrated solution of drugs with 80 μl of Malassezia medium was added to each well of a 96-well microplate, and 180 μl of Malassezia liquid medium alone was added to a well as a negative control; 20 μl of Alamar Blue was added to each well. Sensitivity determinations of the 4 standard strains were performed simultaneously with each test. Each time, the results showed that the minimum inhibitory concentration (MIC) values were in the same range, and the MIC value of each drug varied by no more than one dilution per measurement. The MIC50 value (MIC value of 50% strain inhibition) and MIC90 value (MIC value of 90% strain inhibition) were also determined.

2.4. Sanger sequencing, alignment analysis, and system tree building

Genomic DNA was extracted from the Malassezia strains using the CTAB method [25]. PCR was performed to amplify DNA, and DNA sequencing primers that targeted the D1/D2 region (NL1: 5’-GCA TAT CAA TAA CGG GAG GAA AAG-3’; NL4: 5’-GGT CCG TGT TTC AAG ACG G-3’), and for ITS1-ITS4 region (ITS1: 5’-TCCGTAGGTGAACCTGCGG-3’; ITS4: 5’-TCTTCTCGACTATTAGATGC-3’) were used. The DNA sequences of the determined ITS1-ITS4 regions were spliced by Sequencher 5.4.6 software (Ann Arbor, MI, USA). BLAST and the Genebank database were used to compare similar sequences, and strain were identified based on the highest percentage of bacteria. After the sequences were analyzed by ClustalW2 (Hinxton, Cambridgeshire, United Kingdom), N-J system trees were constructed using Molecular Evolutionary Genetics Analysis (MEGA) software [26].

2.5. Statistical analysis

All statistical analyses were performed using SPSS 17.0 (IBM-SPSS Inc., Chicago, IL, USA). The differences in the distribution of pityriasis versicolor, Malassezia folliculitis and seborrhoeic dermatitis were analyzed by x² tests. The MIC value of each drug was log-transformed and converted for one-way analysis of variance; p < 0.05 indicated that the difference was statistically significant.

3. Results

3.1. Identification of six Malassezia strains isolated from clinical pityriasis versicolor, Malassezia folliculitis and seborrhoeic dermatitis infections

First, the strains were identified by traditional identification methods (morphological, physiological and biochemical characteristics). Among a total of 342 cases, 106 of the 143 patients with pityriasis versicolor had
culture-positive cases (74.1%), 72 of the 96 patients with Malassezia folliculitis had culture-positive cases (75.0%), and 66 of the 103 patients with seborrheic dermatitis had culture-positive cases (64.1%). In total, 244 clinical strains consisting of six Malassezia species were cultured and isolated (71.3%), including 85 strains of M. globosa, 77 strains of M. furfur, 37 strains of M. sympodialis, 28 strains of M. pachydermatis, 10 strains of M. restricta and 7 strains of M. slooffiae, all of which were identified by traditional identification methods. Among them, M. globosa (34.8%), M. furfur (31.6%), M. sympodialis (15.2%), and M. pachydermatis (11.5%) had the highest representation in the three diseases, as shown in Table 1. We also demonstrated that the distribution of strains of the three diseases was significantly different (x² = 33.30, p = 0.000). There was a significant difference between the distribution of pityriasis and Malassezia folliculitis (x² = 20.56, p = 0.000) and between the distribution of Malassezia folliculitis and seborrheic dermatitis (x² = 17.67, p = 0.003). However, there was no significant difference in the distribution of pityriasis versicolor and seborrheic dermatitis (x² = 3.25, p = 0.661). The two main pathogen of pityriasis versicolor and seborrheic dermatitis were M. furfur and M. globosa. The main pathogen of Malassezia folliculitis was M. globosa (59.7%) (Table 1).

3.2. Antifungal effects of four antifungal drugs (ketoconazole, itraconazole, fluconazole and amphotericin B) on Malassezia

Next, we analyzed the antifungal effects of four antifungal drugs (ketoconazole (KTZ), itraconazole (ITZ), fluconazole (FLC) and amphotericin B (Am B)) on 227 strains of Malassezia (M. furfur, M. sympodialis, M. pachydermatis and M. globosa) isolated from clinical samples. The sensitivity of the four Malassezia species to KTZ and ITZ was significantly different (x² = 56.06, p < 0.05). The results showed that the MIC range of KTZ against M. furfur, M. sympodialis, M. pachydermatis and M. globosa strains 0.03 - 0.5 mg/L, 0.03 - 16 mg/L, 0.03 - 0.5 mg/L, and 0.03 - 16 mg/L, respectively (Table 2). As shown in Figure 1A, the MIC values of KTZ against M. furfur, M. pachydermatis and M. globosa for all M. furfur (77/77) and M. pachydermatis strains (28/28) and 89.2% of M. sympodialis strains (33/37) were 0.03 mg/L - 0.5 mg/L (Table 3). The MIC ranges of ITZ against M. furfur, M. sympodialis, M. pachydermatis and M. globosa were 0.03 - 1 mg/L, 0.03 - 0.25 mg/L, 0.03 - 0.25 mg/L, and 0.03 - 0.25 mg/L, respectively (Table 2), although the MIC analysis for ITZ against M. pachydermatis showed two peaks at 0.03 mg/L - 0.13 mg/L and 0.25 mg/L (Figure 1B). The sensitivity of the four Malassezia strains to FLC was significantly different (x² = 85.47, p < 0.05). The MIC ranges of FLC against M. furfur, M. sympodialis, M. pachydermatis and M. globosa were 0.25 - 64 mg/L, 0.25 - 8 mg/L, 0.5 - 16 mg/L, and 0.13 - 16 mg/L, respectively (Table 2). The MIC analysis of FLC against M. furfur showed two peaks at 0.25 - 4 mg/L and 8 - 64 mg/L, at which M. furfur was 62.33% (48/77) and 35.06% (27/77), respectively. The MIC analysis of FLC against M. sympodialis also showed two peaks at 0.25 - 2 mg/L and 2 - 8 mg/L, at which the ratios of M. sympodialis were 72.97% (27/37) and 27.03% (10/37), respectively. The MIC analysis of FLC against M. pachydermatis showed two peaks, with the first peak at 0.5 - 2 mg/L for 53.57% M. pachydermatis (15/28) and the second peak at 4 - 16 mg/L for 46.43% M. pachydermatis (13/28). The MIC of FLC against M. globosa had 2 ranges, with the main peak at 2 - 16 mg/L for 74.12% M. globosa (63/85). The sensitivities of the four Malassezia strains to amphotericin B were significantly different (x² = 124.63, p < 0.05). The MIC ranges of Am B against M. furfur, M. sympodialis, M. pachydermatis and M. globosa were 0.03-8 mg/L, 0.03 - 4 mg/L, 0.03 - 4 mg/L, and 0.03 - 4 mg/L, respectively. The MIC analysis of Am B against M. furfur, M. sympodialis, M. pachydermatis, and M. globosa showed two ranges: 0.06 - 1 mg/L and 2 - 8 mg/L. Approximately 70.27% of M. sympodialis strains (26/37), 85.71% of M. pachydermatis strains (24/28), and 92.94% of M. globosa strains (79/85) had an MIC range of 0.06 - 1 mg/L MIC for Am B; while most M. furfur (70.12%) had MIC ranges from 2 - 8 mg/L for Am B. All the sensitivities of the Malassezia strains to the four antifungal drugs are shown in Figure 1.

The four species of Malassezia were very sensitive to KTZ, but the MIC value of KTZ against M. sympodialis was slightly higher than those against the other species. The percentages of M. furfur, M. sympodialis, M. pachydermatis and M. globosa inhibited by 0.25 mg/L KTZ were 92.21%, 56.76%, 89.29%, and 78.82%, respectively. KTZ activity against these four species of Malassezia was in the following order from strong to weak: M. furfur > M. pachydermatis > M. sympodialis > M. globosa. The four species of Malassezia were very sensitive to ITZ, and the percentages of M. furfur, M. sympodialis, M. pachydermatis and M. globosa inhibited by 0.13 mg/L ITC were 66.2%, 81.21%, 64.28%, and 81.17%, respectively. The antifungal activity of ITZ against these four species of Malassezia ranged from strong to weak in the following order: M. sympodialis > M. globosa > M. furfur > M. pachydermatis. The sensitivity of the four species of Malassezia to FLC was worse than those of Malassezia to the other three drugs. The percentages of M. furfur, M. sympodialis, M. pachydermatis and M. globosa inhibited by 2 mg/L FLC were 51.94%, 96.42%, 53.57%, and 71.76%, respectively. The bacteriostatic action of FLC against these four strains of Malassezia was as follows: M. sympodialis > M. globosa > M. pachydermatis > M. furfur. All four species of Malassezia were more sensitive to Am B than to FLC and were less sensitive to both KTZ and ITZ. In the presence of 1 mg/L Am B, the M. furfur, M. sympodialis, M. pachydermatis and M. globosa strains were inhibited by 29.87%, 78.37%, 92.85%, and 95.29%, respectively. The antifungal activity of Am B against these 4 Malassezia species ranged from strong to weak in the following order: M. globosa > M. pachydermatis > M. sympodialis > M. furfur. The MIC value ranges and the MIC50 and MIC90 values of the four antifungal drugs against the 244 strains of Malassezia are shown in Table 3. The difference in physiological and biochemical characteristics of the Malassezia strains may lead to differences in the activities of antifungal drugs against Malassezia in clinics.

### Table 1. The species of pityriasis, Malassezia folliculitis, and seborrheic dermatitis (N,%).

| Disease                  | M. furfur | M. sympodialis | M. pachydermatis | M. globosa | M. restricta | M. slooffiae | Total   |
|--------------------------|-----------|----------------|------------------|------------|--------------|--------------|---------|
| Pityriasis               | 41 (38.7) | 19 (17.9)      | 15 (14.1)        | 22 (20.7)  | 5 (4.7)      | 4 (3.7)      | 106     |
| Malassezia folliculitis  | 11 (15.2) | 9 (12.5)       | 4 (5.56)         | 43 (59.7)  | 4 (5.56)     | 1 (1.4)      | 72      |
| Seborrheic dermatitis    | 25 (37.8) | 9 (13.6)       | 9 (13.6)         | 20 (30.3)  | 1 (1.5)      | 2 (3.0)      | 66      |
| **Total**                | 77 (31.6) | 37 (15.2)      | 28 (11.5)        | 85 (34.8)  | 10 (4.1)     | 7 (2.9)      | 244     |
versicolor and seborrheic dermatitis \( (p = 0.010) \). However, there was no significant difference in the MIC values of KTZ, ITZ, and Am B against \( M. \) sympodialis from the three diseases \( (p > 0.05) \). There was also no significant difference in the MIC of the four antifungal drugs against \( M. \) pachydermatis or \( M. \) globosa isolated from three diseases \( (p > 0.05) \). Thus, the main treatable species of pityriasis versicolor and seborrheic dermatitis species was \( M. \) furfur, and the best antifungal agent against \( M. \) furfur was KTZ. The Malassezia strains, depending on the different skin diseases they were isolated from, were cultivated and identified when they were collected so that the same type of Malassezia strains were artificially cultivated separately according to different disease sources; this ensures that the same strains from different sources do not contaminate each other during the experiment. The reason why the same strains with different disease origins show different MIC values may be that the strains have different lipase activities \([27, 28]\), suggesting that the formation of lipase activity by the strains may be related to the presence of different sebum concentrations in the skin of the lesion. On another hand, some resistant strains of Malassezia were increasingly detected in different disease sources \([3, 29]\), which may also explain the different sensitivities of the same species of Malassezia to antifungal drugs among the three skin diseases.

3.4. Analysis of the relationship between drug sensitivity and genotypes of the Malassezia strains

Because the phenotypes of Malassezia strains are easily affected by the external environment, traditional identification methods, such as physiological and biochemical methods, can be used to genotype strains and reflect the differences among organisms at the genetic molecular level. We amplified the NL region (B amplification group) to build a phylogenetic tree of Malassezia strains. Among the phylogenetic tree, it can be seen that group 1 (only D17), group 2 (D18, D20, D67, D69, D55, D53, D52, D51, D16, D15, D13, D10, D9, D1, and D7), and group 3 (only D12) formed three relatively independent branches (Figure 2). In addition, the source of strains in group 1 was different from those of the other two groups, and these strains may have evolved from different Malassezia species. We also analyzed the relationship between the drug sensitivities and genotypes of the Malassezia strains in Table 5. However, there was no significant correlation between genotype and drug sensitivity of Malassezia strains from different evolution sources because of the small sample size of analyzed Malassezia strains.

4. Discussion

Due to the special physiological characteristics of Malassezia, except for \( M. \) furfur, the growth of other Malassezia species is lipid dependent, and these strains are unable to grow in media without lipids. Using the fatty acid medium Elegraki RPMI 1640 medium and the National Clinical Trial Committee for Standardization (NCCLS) M27-A2 microdilution method recommended for antifungal drug sensitivity tests, we measured the sensitivities of four species of Malassezia from three diseases to the antifungal drugs FLC, ITZ, KTZ and Am B to provide a better understanding of Malassezia-related diseases in clinics.

### Table 2. MICs (µg/ml) of 7 clinical isolates of Malassezia species to 4 antifungal agents.

| Strain Quantity | KTZ a | ITZ | FLC | Am B |
|-----------------|-------|-----|-----|------|
| MIC range       | MIC50 | MIC90| MIC range | MIC50 | MIC90 | MIC range | MIC50 | MIC90 |
| M. furfur 77    | 0.03–0.5 | 0.13 | 0.25 | 0.03–2.00 | 0.13 | 0.25 | 0.25–64 | 2.00 | 8.00 | 0.03–8 | 2.00 | 4 |
| M. sympodialis 37 | 0.03–16 | 0.25 | 1.00 | 0.03–0.25 | 0.13 | 0.25 | 0.25–8 | 1.00 | 4.00 | 0.03–4 | 0.50 | 4 |
| M. pachydermatis 28 | 0.03–0.5 | 0.13 | 0.50 | 0.03–0.25 | 0.06 | 0.25 | 0.5–16 | 2.00 | 8.00 | 0.03–4 | 0.50 | 1 |
| M. globosa 85 | 0.03–16 | 0.13 | 0.50 | 0.03–0.25 | 0.06 | 0.25 | 0.13–16 | 1.00 | 8.00 | 0.03–4 | 0.25 | 1 |

a KTZ, ketoconazole; ITZ itraconazole; FLC, fluconazole; Am B, amphotericin B (µg ml\(^{-1}\)).

![Figure 1. MIC values of four drugs against four Malassezia species. A) Ketoconazole (KTZ); B) Itraconazole (ITZ); C) Fluconazole (FLC); D) Amphotericin B (AmB).](image-url)
We analyzed the effect of four antifungal drugs on different species of Malassezia, and the MIC_{50} and MIC_{90} values of four Malassezia species to the four antifungal drugs were investigated in 227 Malassezia strains from clinically isolated samples. Among the four antifungal drugs, FLC had the worst antifungal effect against various species of Malassezia. Consist with the vitro MICs values, analysis of the efficacy of ITZ and FLC in the treatment of Malassezia showed that the total effective rate of ITZ for patients was 90.0% (45/50), better than that of FLC for patients (86.0%, 43/50) [30]. At the same time, treatment outcomes of 44 patients diagnosed with Malassezia folliculitis during the 5-year period between March 2007 and October 2013 were analyzed in Japan; the study demonstrated that the treatment was “effective” for all patients, and the mean period required for improvement was 27 ± 16 days for 37 patients receiving the topical application of 2.0% KTZ cream and 14 ± 4 days for the 7 patients receiving 100 mg oral ITZ. The results of topical application of 2.0% KTZ cream alone were similar to those of 100 mg oral ITZ, which is recommended for the treatment of systemic fungal infections caused by Malassezia [31]. KTZ and ITZ, which were superior to FLC and Am B, are recommended for the treatment of pityriasis versicolor and seborrheic dermatitis and had good antifungal effects on Malassezia, however, KTZ can cause toxic necrotizing hepatitis [32]. In a randomized, double-blind, placebo trial that observed both the itraconazole and placebo groups, the itraconazole group showed significant improvement than the placebo group in 57 patients who took 100 mg oral ITZ once daily for 10 days and showed that the effective rate of the itraconazole group was 96.9% (55/57) and the effective rate of the placebo group was 24.6% (14/57) [33].

We analyzed the effect of four antifungal drugs on different species of Malassezia, and the MIC_{50} and MIC_{90} values of four Malassezia species to the four antifungal drugs were investigated in 227 Malassezia strains from clinically isolated samples. Among the four antifungal drugs, FLC had the worst antifungal effect against various species of Malassezia. Consist with the vitro MICs values, analysis of the efficacy of ITZ and FLC in the treatment of Malassezia showed that the total effective rate of ITZ for patients was 90.0% (45/50), better than that of FLC for patients (86.0%, 43/50) [30]. At the same time, treatment outcomes of 44 patients diagnosed with Malassezia folliculitis during the 5-year period between March 2007 and October 2013 were analyzed in Japan; the study demonstrated that the treatment was “effective” for all patients, and the mean period required for improvement was 27 ± 16 days for 37 patients receiving the topical application of 2.0% KTZ cream and 14 ± 4 days for the 7 patients receiving 100 mg oral ITZ. The results of topical application of 2.0% KTZ cream alone were similar to those of 100 mg oral ITZ, which is recommended for the treatment of systemic fungal infections caused by Malassezia [31]. KTZ and ITZ, which were superior to FLC and Am B, are recommended for the treatment of pityriasis versicolor and seborrheic dermatitis and had good antifungal effects on Malassezia, however, KTZ can cause toxic necrotizing hepatitis [32]. In a randomized, double-blind, placebo trial that observed both the itraconazole and placebo groups, the itraconazole group showed significantly higher improvement than the placebo group in 57 patients who took

| Antifungal drug | Pityriasis | Malassezia folliculitis | Seborrheic dermatitis | F     | p     |
|----------------|-----------|------------------------|----------------------|-------|-------|
| **M. furfur**  |           |                        |                      |       |       |
| KTZ            | 0.183 ± 0.020 | 0.060 ± 0.011          | 0.164 ± 0.025        | 7.161 | 0.020^* |
| ITZ            | 0.168 ± 0.021 | 0.148 ± 0.042          | 0.226 ± 0.077        | 0.213 | 0.808 |
| FLC            | 5.226 ± 0.869 | 4.409 ± 1.383          | 6.350 ± 2.477        | 0.324 | 0.270 |
| Am B           | 2.314 ± 0.318 | 1.989 ± 0.746          | 2.396 ± 0.411        | 0.765^* |
| **M. sympodialis** |     |                       |                      |       |       |
| KTZ            | 0.559 ± 0.196 | 0.247 ± 0.557          | 2.181 ± 1.729        | 2.151 | 0.132 |
| ITZ            | 0.112 ± 0.016 | 0.117 ± 0.029          | 0.118 ± 0.028        | 0.055 | 0.947 |
| FLC            | 2.724 ± 0.637 | 1.000 ± 0.415          | 1.278 ± 0.374        | 3.739 | 0.034^* |
| Am B           | 1.058 ± 0.315 | 0.868 ± 0.441          | 1.167 ± 0.538        | 1.207 | 0.312 |
| **M. pachydermatis** |   |                       |                      |       |       |
| KTZ            | 0.210 ± 0.035 | 0.156 ± 0.031          | 0.236 ± 0.039        | 0.641 | 0.535 |
| ITZ            | 0.131 ± 0.023 | 0.172 ± 0.047          | 0.128 ± 0.031        | 0.466 | 0.633 |
| FLC            | 3.068 ± 0.732 | 4.125 ± 1.533          | 3.611 ± 1.628        | 0.664 | 0.523 |
| Am B           | 0.656 ± 0.251 | 0.406 ± 0.094          | 0.490 ± 0.197        | 0.195 | 0.824 |
| **M. globosa** |           |                        |                      |       |       |
| KTZ            | 0.217 ± 0.041 | 0.229 ± 0.026          | 0.184 ± 0.049        | 0.655 | 0.522 |
| ITZ            | 0.126 ± 0.018 | 0.102 ± 0.011          | 0.111 ± 0.019        | 0.583 | 0.560 |
| FLC            | 2.733 ± 0.783 | 2.384 ± 0.469          | 3.175 ± 1.072        | 0.278 | 0.751 |
| Am B           | 0.537 ± 0.189 | 0.494 ± 0.101          | 0.325 ± 0.061        | 0.400 | 0.672 |

Note: “*” indicates statistical significance; “α” indicates one-way ANOVA p value.
itraconazole at 200 mg/daily or a placebo for one week and then the first two days of every month for the following three months [33]. Further studies in patients who responded to these antifungal drugs are essential for correlating in vitro results and investigating whether such clinical outcomes fit with the in vitro MIC values of these drugs. The physiological and biochemical characteristics of the species of Malassezia revealed differences in the sensitivity of the four species of Malassezia to the same antifungal drug.

Among the four species, M. sympodialis and M. globosa were more sensitive to ITZ, while M. furfur and M. pachydermatis were more sensitive to KTZ; such data are similar to the results reported by Hammer [34, 35], although these species have been isolated from different geographical regions. We also demonstrated that all of the Malassezia strains were resistant to FLC in vitro, which is consistent with the results reported by Hammer [34, 35]. In summary, the sensitivity of the Malassezia folliculitis was likely that derived from the other two diseases. Finally, due to the different growth rates of different species of Malassezia, we found that M. furfur and M. pachydermatis grew faster. It was likely that M. furfur and M. pachydermatis competitively inhibited the growth of M. sympodialis and M. globosa, so the strains of M. furfur and M. pachydermatis isolated from the culture might not be all pathogenic or the main pathogens of these diseases when using traditional culture methods. In recent years, some laboratories have applied molecular biological methods to identify the mycelium DNA in samples taken from the skin lesions of patients to avoid traditional culture methods, and these methods can be used to quickly and comprehensively determine the mycelium distribution of Malassezia-related diseases.

In summary, M. furfur, M. sympodialis, M. pachydermatis and M. globosa are the dominant strains causing Malassezia infection in our area. The dominant Malassezia species of Malassezia folliculitis is M. globosa, and the dominant Malassezia species of pityriasis versicolor and seborrheic dermatitis is M. furfur. There are differences in the distribution of dominant species in each disease. The sensitivities of Malassezia to the four

Figure 2. Phylogenetic tree analysis of Malassezia species.

Table 5. MICs (mg/L) values of KTZ, ITZ, FLC, and AmB against Malassezia strains, and the molecular identification of the organisms.

| Strain No. | KTZ      | ITZ      | FLC      | AmB      | NL identification | ITS identification | Traditional identification | Identification Group No. |
|-----------|----------|----------|----------|----------|-------------------|----------------------|---------------------------|------------------------|
| D20       | 0.125    | 0.250    | 2.000    | 2.000    | Malassezia furfur  | Malassezia furfur    | Malassezia sympodialis    | 2                      |
| D1        | 0.125    | 0.125    | 2.000    | 1.000    | Malassezia furfur  | -                    | Malassezia globosa        | 2                      |
| D11       | 0.031    | 0.063    | 1.000    | 0.031    | Malassezia furfur  | Malassezia furfur    | Malassezia pachydermatis  | 2                      |
| D9        | 0.125    | 0.250    | 8.000    | 2.000    | Malassezia furfur  | -                    | Malassezia furfur         | 2                      |
| D12       | 0.250    | 0.500    | 8.000    | 2.000    | Malassezia furfur  | -                    | Malassezia furfur         | 3                      |
| D13       | 0.125    | 0.125    | 1.000    | 4.000    | Malassezia furfur  | -                    | Malassezia furfur         | 2                      |
| D14       | 0.500    | 0.125    | 0.500    | 8.000    | Malassezia furfur  | -                    | Malassezia furfur         | 2                      |
| D16       | 0.250    | 0.500    | 8.000    | 2.000    | Malassezia furfur  | Malassezia furfur    | Malassezia furfur         | 2                      |
| D18       | 0.250    | 0.250    | 16.000   | 0.500    | Malassezia furfur  | Malassezia furfur    | Malassezia furfur         | 2                      |
| D38       | 0.500    | 0.250    | 4.000    | 0.500    | Malassezia furfur  | Malassezia furfur    | Malassezia globosa        | 2                      |
| D51       | 0.031    | 0.063    | 1.000    | 0.063    | Malassezia furfur  | Malassezia furfur    | Malassezia restricta      | 2                      |
| D52       | 0.031    | 0.125    | 2.000    | 0.063    | Malassezia furfur  | Malassezia furfur    | Malassezia globosa        | 2                      |
| D53       | 0.500    | 0.063    | 2.000    | 1.000    | Malassezia furfur  | Malassezia furfur    | Malassezia globosa        | 2                      |
| D55       | 0.031    | 0.063    | 1.000    | 0.063    | Malassezia furfur  | Malassezia furfur    | Malassezia globosa        | 2                      |
| D69       | 0.250    | 0.500    | 8.000    | 2.000    | Malassezia furfur  | Malassezia furfur    | Malassezia furfur         | 2                      |
| D7        | 0.125    | 0.250    | 8.000    | 2.000    | Malassezia furfur  | Malassezia furfur    | Malassezia furfur         | 2                      |
| D67       | 0.250    | 0.125    | 1.000    | 2.000    | Malassezia furfur  | Malassezia furfur    | Malassezia furfur         | 2                      |
| D17       | 0.250    | 0.0625   | 0.250    | 0.500    | Malassezia furfur  | -                    | Malassezia sympodialis    | 1                      |
antifungal drugs were ITZ > KTZ > Am B > FLC. M. furfur from three diseases had a different sensitivity to KTZ. Our results showed that KTZ and ITZ had the best antifungal activity against Malassezia, but KTZ can cause toxic necrotizing hepatitis. Therefore, it is recommended that for the treatment of Malassezia in the clinic, ITZ is preferred as the drug of choice. In the future, the identification and drug susceptibility testing of various species of Malassezia should be performed, which will improve the treatment of these skin diseases in the clinic.

Declarations

Author contribution statement

J. Wang and Y. Li: Contributed reagents, materials, analysis tools or data.
K. Wang: Performed the experiments; Wrote the paper.
L. Cheng and W. Li: Performed the experiments.
H. Jiang: and X. Zhang: Analyzed and interpreted the data; Wrote the paper.
S. Liu, Y. Huang, Q. Mingye and T. Dong: Analyzed and interpreted the data.
S. Feng: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.
H. Li: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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