Spread of Terbinafine-Resistant *Trichophyton mentagrophytes* Type VIII (India) in Germany—“The Tip of the Iceberg?”

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Abstract: Chronic recalcitrant dermatophytoses, due to *Trichophyton* (*T.*) *mentagrophytes* Type VIII are on the rise in India and are noteworthy for their predominance. It would not be wrong to assume that travel and migration would be responsible for the spread of *T. mentagrophytes* Type VIII from India, with many strains resistant to terbinafine, to other parts of the world. From September 2016 until March 2020, a total of 29 strains of *T. mentagrophytes* Type VIII (India) were isolated. All patients were residents of Germany: 12 females, 15 males and the gender of the remaining two was not assignable. Patients originated from India (11), Pakistan (two), Bangladesh (one), Iraq (two), Bahrain (one), Libya (one) and other unspecified countries (10). At least two patients were German-born residents. Most samples (21) were collected in 2019 and 2020. All 29 *T. mentagrophytes* isolates were sequenced (internal transcribed spacer (ITS) and translation elongation factor 1-α gene (*TEF1-α*)). All were identified as genotype VIII (India) of *T. mentagrophytes*. In vitro resistance testing revealed 13/29 strains (45%) to be terbinafine-resistant with minimum inhibitory concentration (MIC) breakpoints ≥0.2 µg/mL. The remaining 16 strains (55%) were terbinafine-sensitive. Point mutation analysis revealed that 10/13 resistant strains exhibited Phe<sup>397</sup>Leu amino acid substitution of squalene epoxidase (SQLE), indicative for in vitro resistance to terbinafine. Two resistant strains showed combined Phe<sup>397</sup>Leu and Ala<sup>448</sup>Thr amino acid substitutions, and one strain a single Leu<sup>393</sup>Phe amino acid substitution. Out of 16 terbinafine-sensitive strains, in eight Ala<sup>448</sup>Thr, and in one Ala<sup>448</sup>Thr + new Val<sup>444</sup> Ile amino acid substitutions were detected. Resistance to both itraconazole and voriconazole was observed in three out of 13 analyzed strains. Treatment included topical ciclopirox olamine plus topical miconazole or sertaconazole. Oral itraconazole 200 mg twice daily for four to eight weeks was found to be adequate. Terbinafine-resistant *T. mentagrophytes* Type VIII are being increasingly isolated. In Germany, transmission of *T. mentagrophytes* Type VIII from the Indian subcontinent to Europe should be viewed as a significant public health issue.

Keywords: dermatophytoses; terbinafine-resistant; squalene epoxidase; point mutation; transmission; Itraconazole; Ciclopirox; Miconazole

1. Introduction

There is a veritable epidemic of varieties of chronic recalcitrant dermatophytoses due to *Trichophyton* (*T.*) *mentagrophytes* Type VIII in India [1]. A wide variation in clinical features is seen.
Tinea corporis, tinea cruris, tinea faciei and their combinations are the most common presentations. Lesions often show a minimal to a high degree of inflammation, and large lesions with a tendency to coalesce and spread are common. Severe itching is common [2]. There has been an undeniable association between the occurrence of extensive and hard to treat tinea and long-term abuse of potent and super-potent topical corticosteroids, predominantly clobetasol propionate [1,3].

Extensive travel and migration are considered vital in the spread of dermatophytoses. Especially the terbinafine-resistant strains of *T. mentagrophytes* Type VIII, detected as the causative genotype, are now increasingly isolated in Germany and other European countries. The main criterion to identify this particular infection is the very noticeable treatment failure with topical and oral terbinafine.

2. Patients and Methods

2.1. Patients

Patients discussed herein were predominantly German residents with chronic dermatophytoses who had been failing treatment with terbinafine and, therefore, were suspected to harbour *T. mentagrophytes* Type VIII. They were subjected to mycological diagnostics including Blancophor® preparation, fungal culture and molecular biological fungal DNA detection. Skin scrapings taken from suspicious skin sites were investigated. Additionally, a few fungal cultures isolated in other laboratories were sent to our center for precise identification of the fungal species and internal transcribed spacer (ITS) genotype has been included in the epidemiological investigation (Table 1).

2.2. Conventional Cultural Diagnostics

In mycological routine diagnostics, scrapings from the active edges of centrifugally spreading lesions of the free skin, as well as hair roots from lesions of the capillitium in some patients, were cultured on Sabouraud’s 4% dextrose agar (Sifin, Berlin, Germany) and, additionally, on cycloheximide (Actidione®-containing Sabouraud’s dextrose agar, Becton Dickinson, Heidelberg, Germany). Fungal isolates showing fast-growing, flat radiating fungal colonies with a white periphery, and sometimes bright yellowish centre typical for *T. mentagrophytes*, were further analyzed. Microscopic lactophenol cotton blue preparations were performed from such colonies.

2.3. Molecular Biological Characteristics of Trichophyton mentagrophytes ITS Type VIII

2.3.1. PCR-ELISA for Molecular Identification of Dermatophytes

DNA from either skin scrapings or fungal isolates (for identification of submitted fungal cultures) was extracted according to the manufacturer’s protocol using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany). Samples were analyzed using a validated and standardized in-house developed enzyme linked immunoassay (PCR-ELISA) to detect dermatophyte DNA [4,5]. Specific probes detecting the following relevant dermatophytes were used: *T. rubrum*, *T. interdigitale/T. mentagrophytes*, *Microsporum canis*, and *T. benhamiae* (formerly referred to as *T. anamorph* or *Arthroderma benhamiae*).

All DNA samples extracted either from skin scrapings or from fungal cultures were positive in the PCR-ELISA for *T. interdigitale/T. mentagrophytes*. As the differentiation between *T. interdigitale* and the *T. mentagrophytes* complex was not possible by PCR-ELISA, the ITS regions of rDNA genes and the translation elongation factor (TEF)1-α gene were sequenced.
Table 1. Overview of the 29 patients with dermatomycoses due to *T. mentagrophytes* of (ITS) genotype VIII (India) diagnosed all over Germany from 2016–2020. Abbreviations: SQLE, squalene epoxidase; ITS, internal transcribed spacer; TEF1-α, translation elongation factor 1-α; MIC, minimum inhibitory concentration; NCBI, National Center for Biotechnology Information, Bethesda, Maryland; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany; f, female; m, male.

| Strain Number | Collection No. | Gender | Age in Years/Months | Pathology | Additional Information/Remarks | MIC Terbinafine [µg/mL] | Drug Resistance to Terbinafine | Amino Acid Substitution within the Squalene Epoxidase | Codon Change in SQLE | MIC Itraconazole [µg/mL] | Drug Resistance to Itraconazole | MIC Voriconazole [µg/mL] | Drug Resistance to Voriconazole | GenBank Accession Number | GenBank Accession Number TEF1-α Gene (NCBI) | GenBank Accession Number SQLE Gene (NCBI) | Sample Date |
|---------------|----------------|--------|---------------------|-----------|--------------------------------|------------------------|-----------------------------|----------------------------------|-----------------|------------------------|----------------------------------|----------------|--------------------------|----------------------|--------------------------|-------------------------------|----------------|
| 1 21467 7/16  | DSM 108903     | f      | 35                  | Tinea pedis | Indian                          | 0.2                    | resistant                    | Phe397Leu                        | TTC → CTC | 0.06                   | sensitive                        | 0.03             | sensitive                 | MT330252              | MT340499                  | MT700499                    | 2016          |
| 2 21637 7/17  | DSM 108902     | f      | 29                  | Tinea       | Foreigners                      | 0.2                    | resistant                    | Phe397Leu                        | TTC → CTC | 0.06                   | sensitive                        | 0.03             | sensitive                 | MT330249              | MT340500                  | MT700500                    | 2017          |
| 3 21154 2/18  | DSM 108901     | m      | 19                  | Tinea corporis | Asylum office Herford          | 8                      | resistant                    | Phe397Leu                        | TTC → CTC | 0.087                  | sensitive                        | 0.088            | sensitive                 | MT330250              | MT340501                  | MT700501                    | 2018          |
| 4 21156 4/18  | DSM 108900     | f      | 30                  | Tinea unguium et corporis       | Indian              | <0.2                  | sensitive                    | Ala448Thr                        | GCT → ACT | 0.067                  | sensitive                        | 0.088            | sensitive                 | MT330248              | MT340502                  | MT700502                    | 2018          |
| 5 21816 1/18  | DSM 108899     | m      | 35                  | Tinea corporis et cruris        | Indian              | <0.2                  | sensitive                    | wild type                        | -                | 0.125                  | sensitive                        | 0.03             | sensitive                 | MT330253              | MT340503                  | MT700503                    | 2018          |
| 6 21836 1/18  | DSM 108898     | f      | 25                  | Tinea corporis                   | married couple on a pilgrimage in Saudi Arabia | <0.2                  | sensitive                    | Ala448Thr                        | GCT → ACT | 0.5                    | resistant                        | 0.5              | resistant                 | MT330255              | MT340504                  | MT700504                    | 2018          |
| 7 21869 1/18  | DSM 108897     | m      | 58                  | Tinea facies                     | Visit India         | 8                      | resistant                    | Phe397Leu                        | TTC → CTC | 0.125                  | sensitive                        | 0.03             | sensitive                 | MT330254              | MT340505                  | MT700505                    | 2018          |
| 8 90153 8/18  | DSM 108896     | m      | 4                   | Tinea corporis                   | German name         | <0.2                  | sensitive                    | Ala448Thr                        | GCT → ACT | 0.25                   | sensitive                        | 0.25             | resistant                 | MT330251              | MT340506                  | MT700506                    | 2018          |
| 9 20061 8/19  | DSM 109024     | f      | 6 months            | Tinea corporis                   | Family came from Bahrain to visit Germany | 0.2                    | resistant                    | Phe397Leu                        | TTC → TTA | not applied            | not applied                       | -                | not applied               | MT330285              | MT345568                  | MT700507                    | 2019          |
| 10 20295 3/19 | DSM 109747     | m      | 27                  | Tinea                             | Libyan, for three years in Germany, German girlfriend also affected | 8                      | resistant                    | Phe397Leu                        | TTC → CTC | 0.5                    | resistant                        | 0.25             | resistant                 | MT330284              | MT340507                  | MT700508                    | 2019          |
| Strain Number | Collect ion No. | Gender | Age in Years/ Months | Pathology | Additional Information/ Remarks | MIC Terbina fine [µg/mL] | Drug Resistance to Terbina fine | Amino Acid Substitution within the Squalene Epoxidase | Codon Change in SQLE | MIC Itracozole [µg/mL] | Drug Resistance to Itracozole | MIC Voriconazole [µg/mL] | Drug Resistance to Voriconazole | GenBank Accession Number ITS Gene | GenBank Accession Number TEF1-α Gene (NCBI) | GenBank Accession Number SQUE Gene (NCBI) | Sample Date |
|---------------|----------------|--------|----------------------|-----------|----------------------------------|-------------------------|-------------------------------|--------------------------------------|-------------------|------------------|--------------------------------------|-----------------|--------------------------------------|---------------------------------|------------------------------------------|----------------------------------------|-------------|
| 11            | 20351           | f      | 25                   | Tinea cruris et inguinialis | Pakistani | <0.2 sensitive wild type         | -                        | 0.0312 sensitive               | 0.0312 sensitive | MT330280            | MT340508                        | MT700509 | 2019                                |
| 12            | 60017           | f      | 34                   | Tinea corporis             | Indian, was in India when the skin symptoms started | <0.2 sensitive Ala448Thr | GCT → ACT                   | 0.125 sensitive              | 0.0312 sensitive | MT330279            | MT340517                        | MT700510 | 2019                                |
| 13            | 20566           | m      | 32                   | Tinea cruris               | Iraqi couple (see no. 14) | <0.2 sensitive Ala448Thr | GCT → ACT                   | 0.125 sensitive              | 0.125 sensitive | MT330281            | MT340516                        | MT700511 | 2019                                |
| 14            | 20566           | f      | 31                   | Tinea corporis             | Iraqi couple (see no. 13) | <0.2 sensitive Ala448Thr | GCT → ACT                   | 0.5 resistant                | 0.5 resistant | MT330283            | MT340515                        | MT700512 | 2019                                |
| 15            | 20873           | f      | 29                   | Tinea corporis             | Indian name                  | 8 resistant Phe397Leu      | TTC → TTA not applied       | -                         | not applied          | MT328783                        | MT340514 | MT700513 | 2019                                |
| 16            | 20993           | m      | 28                   | Tinea corporis gluteal     | Indian name                  | <0.2 sensitive Ala448Thr   | GCT → ACT                   | not applied                | not applied          | MT330268                        | MT340510 | MT700515 | 2019                                |
| 17            | 60023           | m      | 40                   | Tinea corporis             | Family, large plaques       | 16 resistant Leu393Phe     | TTC → TIC not applied       | -                         | not applied          | MT330279                        | MT340512 | MT700515 | 2019                                |
| 18            | 21417           | m      | 37                   | Tinea faciei and corporis: cheeks, neck, forehead, and ear helix | German | 8 resistant Phe397Leu Ala448Thr | TTC → CTC GCT → ACT not applied | -                         | not applied          | MT330289                        | MT340511 | MT700516 | 2019                                |
| 19            | 21653           | m      | 27                   | Tinea corporis             | Indian                        | <0.2 sensitive Ala448Thr | GCT → ACT                   | not applied                | not applied          | MT330288                        | MT340510 | MT700517 | 2019                                |
| 20            | 21720           | m      | 24                   | Tinea corporis             | Indian                        | <0.2 sensitive Val444 Ile Ala448Thr | GTA → ATA GCT → ACT not applied | -                         | not applied          | MT330266                        | MT340509 | MT700518 | 2019                                |
| 21            | 21967           | m      | 20                   | Tinea corporis             | Foreigner                     | <0.2 sensitive wild type  | -                         | not applied                | not applied          | MT330256                        | MT340518 | MT700519 | 2019                                |
| 22            | 21923           | f      | 27                   | Tinea corporis             | Pakistani                     | <0.2 sensitive wild type  | -                         | not applied                | not applied          | MT330290                        | MT340519 | MT700520 | 2019                                |
Table 1. Cont.

| Strain Number | Collection No. | Gender | Age in Years/ Months | Pathology | Additional Information/ Remarks | MIC Terbinafine [µg/mL] | Drug Resistance to Terbinafine | Amino Acid Substitution within the Squalene Epoxidase | Codon Change in SQLE | MIC Itraconazole [µg/mL] | Drug Resistance to Itraconazole | MIC Voriconazole [µg/mL] | Drug Resistance to Voriconazole | GenBank Accession Number TEF1-α Gene (NCBI) | GenBank Accession Number SQLE Gene (NCBI) | GenBank Accession Number ITS Gene | Sample Date |
|---------------|----------------|--------|----------------------|-----------|---------------------------------|-------------------------|-----------------------------|------------------------------------------------|-----------------|--------------------------|---------------------------------|--------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|-------------|
| 23            | 60038          | -      | -                    | Tinea     | Indian                          | <0.2 sensitive         | wild type                   | -                                   | not applied     | -                        | not applied                     | -                        | -                                | MT330291                        | MT340520                        | MT700521                        | 2019         |
| 24            | 220577         | -      | f 24                 | Tinea corporis gluteal | Student from Bangladesh | <0.2 sensitive | wild type | -                                   | not applied | -                        | not applied                     | -                        | -                                | MT330267                        | MT340521                        | MT700522                        | 2019         |
| 25            | 60000          | -      | m                    | Tinea cruris | German patient with stay in Thailand and India | 16 resistant | Phe397Leu TTC → CTC | not applied | -                        | not applied                     | -                        | -                                | MT333227                        | MT340522                        | MT700523                        | 2020         |
| 26            | 10154          | -      | m 30                 | Tinea     | Foreigner                       | 16 resistant | Phe397Leu Ala448Thr TTC → CTC GCT → ACT | not applied | -                        | not applied                     | -                        | -                                | MT333225                        | MT340523                        | MT700524                        | 2020         |
| 27            | 20067          | -      | m 27                 | Tinea corporis et manum | Foreigner | <0.2 sensitive | wild type | -                                   | not applied | -                        | not applied                     | -                        | -                                | MT333228                        | MT345569                        | MT700525                        | 2020         |
| 28            | 90013          | -      | m 51                 | Tinea corporis | Originating and migrating for studying from Bangladesh, her father also affected. She was pretreated in her home country by oral voriconazole, without success. | 16 resistant | Phe397Leu TTC → CTC | not applied | -                        | not applied                     | -                        | -                                | MT333226                        | MT340524                        | MT700526                        | 2020         |
| 29            | 20453          | -      | f 23                 | Tinea corporis | 0.2 resistant | Phe397Leu TTC → CTC | not applied | -                        | not applied                     | -                        | -                                | MT333242                        | MT340525                        | MT700527                        | 2020         |
2.3.2. Sequencing of the ITS Regions of rDNA Genes for Species Identification of *Trichophyton mentagrophytes* Type VIII

For confirmation of the suspected dermatophyte species, Sanger sequencing of the ITS regions of rDNA genes (mainly the regions ITS 1, 5.8 S rRNA, ITS 2) and *TEF1*-α gene was performed for all isolates [6–9]. This required PCR amplification of a ~900 bp DNA fragment using universal primers that bind to flanking pan-fungal sequence regions: V9G (5’-TTACGTCCCTGCCCTTTGTA-3’) and LS266 (5’-GCATTCCCAAACAACGTGACTC-3’).

The length of the analyzed region in the *TEF1*-α gene varied from 709 to 769 nucleotides among the various dermatophyte species. Primers EF1a-F (5’-CACATTAACTTGGTCGTTATCG-3’) and EF1a-R (5’-CATCCCTTGAGATACCAAGC-3’) were used for sequencing [6].

2.3.3. Phylogenetic Analysis of *Trichophyton mentagrophytes* Type VIII

Both reference strains and clinically isolated wild type strains were used for comparative molecular analysis, and the generation of the phylogenetic tree based on the ITS region and the TEF 1α gene is listed in Table 2. In addition, GenBank numbers of all sequences used for generating phylogenetic trees are provided in Table 2.

### Table 2. Reference strains and clinical isolates used to generate the phylogenetic tree based on sequencing of the ITS region of rDNA and the TEF1-α gene. GenBank accession numbers of the nucleotide sequences used in this study are available at the NCBI. Abbreviations: NCBI, National Center for Biotechnology Information, Bethesda, Maryland; ITS, internal transcribed spacer; rDNA, ribosomal DNA; TEF1-α, translation elongation factor 1-α; CBS, Centraal Bureau voor Schimmelcultures (today, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands); DSM, *T. mentagrophytes* Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany; ATCC, American Type Culture Collection (Manassas, VA, USA). *means a subtype of the genotype II.

| Species/Genotype | Strain | Collection | Accession No. NCBI ITS | Accession No. NCBI TEF1-α |
|------------------|--------|------------|-----------------------|--------------------------|
| *T. interdigitale* ITS genotype I | 208223/17 | DSM 108620 | MK447595 | MK460538 |
| *T. interdigitale* ITS genotype I deposited in the NCBI as *T. interdigitale* | Isolate 2 | KC595991 |
| *T. interdigitale* ITS genotype II | 200070/17 | DSM 108621 | MK447596 | MK460539 |
| *T. interdigitale* ITS genotype II deposited in the NCBI as *T. interdigitale* | RCPF-1301 | KX308373 |
| *T. interdigitale* ITS genotype II deposited in the NCBI as *T. interdigitale* | CBS 475.93 | MF926357 |
| *T. interdigitale* ITS genotype II deposited in the NCBI as *T. interdigitale* | CBS 428.63 | NR_144900 |
| *T. interdigitale* ITS genotype II deposited in the NCBI as *T. mentagrophytes* | CBS 647.73 | KT15955 |
| *T. mentagrophytes* deposited in the NCBI as *T. mentagrophytes* | CBS 102.68 | KM678062 |
| *T. interdigitale* ITS genotype II * | 212063/17 | DSM 108905 | MK630684 | MK751367 |
| *T. interdigitale* ITS genotype II * | 250016/18 | DSM 108630 | MN866818 | MN886231 |
| *T. mentagrophytes* | CBS 116916 | KM678130 |
| *T. interdigitale* | CBS 130940 | KM678173 |
| *T. mentagrophytes* ITS genotype III | 217704/15 | DSM 108630 | MK453025 | MK460541 |
| *T. mentagrophytes* ITS genotype III | 200026/16 | DSM 103451 | KX866689 | MK460540 |
Table 2. Cont.

| Species/Genotype | Strain     | Collection  | Accession No. NCBI ITS | Accession No. NCBI TEF1-α |
|------------------|------------|-------------|------------------------|--------------------------|
| ITS genotype III deposited in the NCBI as *T. cf. mentagrophytes* | ATCC 60612 | KJ606099     |                        |                          |
| *T. mentagrophytes* ITS genotype III deposited in the NCBI as *T. mentagrophytes* | RCPF-1207 | KT253559     |                        |                          |
| *T. mentagrophytes* ITS genotype III * | 217907/15 | DSM 108628 | MK447605 | MK460542               |
| *T. mentagrophytes* ITS genotype III * | 218893/16 | DSM 108629 | MK447604 | MK460543               |
| *T. mentagrophytes* ITS genotype III * | 900120/17 | DSM 108632 | MK447606 | MK460544               |
| *T. mentagrophytes* ITS genotype III * deposited in the NCBI as *T. interdigitale* | CZE 4473 | LN736306     |                        |                          |
| *T. mentagrophytes* ITS genotype IV deposited in the NCBI as *T. mentagrophytes* | CBS 304.38 | MF926360     |                        |                          |
| *T. mentagrophytes* ITS genotype V deposited in the NCBI as *T. cf. mentagrophytes* | ATCC 46950 | KJ606098     |                        |                          |
| *T. mentagrophytes* ITS genotype V | 600014/20 | DSM 108625 | MK450323 | MK467450               |
| *T. mentagrophytes* ITS genotype V | 600024/20 | DSM 108622 | MK450322 | MK467448               |
| *T. mentagrophytes* ITS genotype V | 600184/19 | DSM 108623 | MK447611 | MK460545               |
| *T. mentagrophytes* ITS genotype V | 600197/19 | DSM 108624 | MK450324 | MK467449               |
| *T. mentagrophytes* ITS genotype V | 600316/19 | DSM 108625 | MK450324 | MK467449               |
| *T. mentagrophytes* ITS genotype V | 600316/19 | DSM 108625 | MK450324 | MK467449               |
| *T. mentagrophytes* ITS genotype VI | 218904/16 | DSM 108622 | MK450322 | MK467448               |
| *T. mentagrophytes* ITS genotype VI | 200128/17 | DSM 108623 | MK447611 | MK460545               |
| *T. mentagrophytes* ITS genotype VI | 215003/16 | DSM 108624 | MK450324 | MK467449               |
| *T. mentagrophytes* ITS genotype VII | 211509/17 | DSM 108625 | MK450323 | MK467450               |
| *T. quinckeanum* | 216686/15 | DSM 107597 | MH791420 | MH802491               |
| *T. quinckeanum* | 210314/16 | DSM 107597 | MH791420 | MH802491               |
| *T. quinckeanum* | 218251/16 | DSM 107597 | MH791420 | MH802491               |
| *T. quinckeanum* | 210314/16 | DSM 107597 | MH791420 | MH802491               |
| *T. quinckeanum* | 216686/15 | DSM 107597 | MH791420 | MH802491               |

2.3.4. Statistical Method for Generating Phylogenetic Trees

Phylogenetic relationships between dermatophyte species were generated using the software Mega X: Analysis Statistical Method Maximum Likelihood, Phylogeny Test Bootstrap Method Replications–1000, Substitution Model Maximum Composite Likelihood [10,11].

2.3.5. Deposition of the Isolates in Strain Collections and Gene Databases

Both ITS and TEF1α gene sequences of all 29 strains/isolates are deposited at the database of the National Centre for Biotechnology Information (NCBI) in Bethesda, MD, USA (Table 1). The strains themselves were deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

2.4. Antifungal Resistance Testing

2.4.1. In Vitro Antifungal Susceptibility Testing

Isolated dermatophytes growing on culture media were tested for growth on Sabouraud’s dextrose agar containing 0.2 µg/mL terbinafine, as described by previous research [12]. The concentration of terbinafine was equivalent to twice that of the minimal inhibitory concentration (MIC) for T. mentagrophytes and T. rubrum under these conditions [13]. Fungal growth was examined after seven and 14 days. Growing strains were recorded as resistant. MICs of itraconazole and voriconazole were determined according to the broth microdilution method of the Clinical and Laboratory Standards Institute as previously described [14]. Based on epidemiological cut-off values (ECOFFs) from previous research, strains were classified as resistant or sensitive to itraconazole and voriconazole (ECOFF ≥ 0.5 µg/mL for itraconazole; ECOFF ≥ 0.25 µg/mL for voriconazole) [15].

2.4.2. Squalene Epoxidase Gene Analysis

Trichophyton total DNA was extracted from fresh fungal cultures on Sabouraud’s dextrose agar using a DNeasy Plant Minikit (Qiagen, Hilden, Germany). A square shaped area of approximately 1.0 mm² of growing culture was used. The squalene epoxidase (SQLE) gene of the terbinafine-resistant clinical isolates was amplified by PCR with ReadyMix Taq PCR Reaction Mix (Sigma Aldrich, Merck). The primer pair TrSQLE-F2 (5’ ATGGTTGTAGAGGCTCCTCCC 3’) and TrSQLE-R1 (5’ CTAGCTTTGAAAGTTCGGCAAA 3’) was used and chromosomal DNA served as the template. In some cases where fungal cultures were not obtained, SQLE gene fragments were analyzed from scale DNA as described [16] using primer pairs TmSQLEF4 (5’ AACGGCTTTGCGAATGGCTCC 3’) and TmSQRLE4 (5’ GATGACCCTGCCAGCAGTAAAG 3’). Sequences were aligned and screened for missense mutations using MEGA version 10.0.5 [10,11].

3. Results

3.1. Patients

Twenty-nine patients (all out-patients) with different clinical variants of dermatophytoses caused by T. mentagrophytes Type VIII (India) were diagnosed all over Germany (Table 1) between September 2016 and March 2020. The detection was based on both routine diagnostics performed in the laboratory Mölbis, Germany, and from cultures sent for fungal species identification of T. mentagrophytes Type VIII (India). The microbiological as well as molecular diagnosis of T. mentagrophytes Type VIII was possible in all 29 patients.

Males (n = 15) outnumbered females (n = 12). Gender was not specified for two patients. Patients’ age ranged from six months to 58 years. The mean age was 26 years (n = 26, for three patients the age was not known). Patients included two children aged six months and four years. Tinea corporis was the predominant variant of dermatophytosis (n = 14). Other variants of dermatophytoses were unspecified tinea (n = 3), tinea corporis and tinea cruris (n = 3), tinea cruris (n = 2), tinea corporis
(including tinea glutealis) \( n = 2 \), tinea faciei \( n = 2 \), tinea corporis and tinea unguium \( n = 1 \), tinea pedis \( n = 1 \), and tinea corporis and tinea manuum \( n = 1 \) (Table 1, Figure 1a,b). Both toddlers presented with tinea corporis (including tinea glutealis).

Figure 1. (a) Tinea corporis due to \( T. \) mentagrophytes VIII in a 34-year old female from India residing in Germany. Patient No. 12. Laboratory number of the fungal isolate: 600174/2019. (b) Accompanying large area tinea of the thighs of the same patient. (c) Subculture of \( T. \) mentagrophytes VIII isolated from the patient’s skin scrapings on Sabouraud’s dextrose agar; developed fast growing white, flat, radiating and granular colonies.

3.2. Some Striking Clinical Presentations

(1) A six months-old-female infant from Bahrain visiting Germany with her family for a holiday was seen by us for extensive dermatophytosis of the back, buttocks, chest and groin [17]. Topical treatment by terbinafine for over two months was unsuccessful. Other family members, including adults and children, were treated in Bahrain with topical antifungals and oral voriconazole which was not helpful. The girl was successfully treated by topical miconazole and later by ciclopirox olamine.

(2) A 28-year old male from Libya, living for three years in Germany, suffered from tinea cruris and tinea faciei involving the left upper and lower eyelids (Figure 2a,b). Treatment by oral fluconazole and terbinafine had failed. His German girlfriend was also affected by the dermatophytosis, though her child was spared. The patient had no contact with India, Indians or Arabs and had not visited Libya in the past few years. The man, however, regularly went to the gym. Treatment with itraconazole orally 400 mg daily for eight weeks cured him.
Figure 2. (a) Tinea cruris due to *T. mentagrophytes* VIII in a 28-year old male from Libya residing in Germany for about three years. Patient No. 10. Laboratory number of the fungal isolate: 202953/2019. (b) Accompanying tinea faciei of the same patient involving the left upper and lower eyelids.

(3) A pregnant German woman presented with tinea cruris et corporis after a trip to Saudi Arabia. Her husband was also affected. Topical treatment was started by clotrimazole alone.

(4) An Iraqi couple living in Germany for a long time suffered from chronic recalcitrant dermatophytosis of the groin, thighs and buttocks for at least two years (Figure 3). Repeated topical treatments by fixed-dose combination creams (FDCs), also known as combination creams, (fluprednidene 21-acetate + miconazole nitrate, betamethasone dipropionate + gentamicin sulphate, and betamethasone dipropionate + clotrimazole) had failed. Topical antifungal therapy (ciclopirox olamine, sertaconazole) given for five to six weeks acted very slowly and they stopped treatment due to progress of the disease. Oral itraconazole 200 mg daily was started for four weeks leading to resolution.

Figure 3. Tinea corporis und tinea glutealis due to *T. mentagrophytes* VIII in a woman originating from Iraq and living for a long time in Germany. Typical inflammatory and itching erythematousquamous plaques were observed involving the buttocks, the groin and the thighs. Patient No. 14. Laboratory number of the fungal isolate: 205667/2019.

Phylogenetic analysis of Trichophyton mentagrophytes Type VIII in comparison to other genotypes. Species identification was confirmed for all 29 isolates by sequencing of the ITS regions of rDNA genes. Molecular relationships of these 29 isolates with other genotypes within the species *T. mentagrophytes*, and with closely related dermatophytes, were depicted in a phylogenetic tree/dendrogram of the sequences (Figure 4a). All 29 isolates belonged to the same cluster, called ITS Type VIII, referred to as Indian variant. The isolates of *T. mentagrophytes* ITS Type VIII formed
their own phylogenetic cluster. This genotype was clearly different from other already known genotypes of *T. mentagrophytes*, e.g., zoophilic strains isolated from human dermatophytoses and from animals, including a snow leopard at a zoo garden, and from *T. mentagrophytes* ITS genotype VII (Thai variant). The anthropophilic *T. interdigitale* could be distinguished clearly from zoophilic *T. mentagrophytes* clusters.

Figure 4. Cont.
Figure 4. Phylogenetic analysis of the dermatophyte isolates based on the ITS regions of rDNA and the TEF1-α gene (Table 2). The evolutionary history was inferred by using the maximum likelihood method and Tamura-Nei model [10]. The tree with the highest log likelihood (−907.70) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 28 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. There were a total of 1086 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [11]. (a) Phylogenetic tree of T. mentagrophytes based on sequencing of the ITS regions of rDNA genes. By sequencing, a 100% concordance with NCBI reference strains (accession numbers MH791420, MH791425, MH990852) was found for all 29 isolates. All these isolates formed their own cluster, which is
now called the ITS genotype VIII (India) of *T. mentagrophytes*. These isolates (*T. mentagrophytes* Type VIII or clade) were clearly discriminated from already known *T. mentagrophytes* genotypes, e.g., II, V, VII. Rooted with *Trichophyton quinckeanum*. (b) Phylogenetic tree of *T. mentagrophytes* based on sequencing of the TEF1-α gene. Used NCBI reference sequences (TEF1-α gene) were MH802491 and MH802496 (accession numbers). Within the phylogenetic tree, all *T. mentagrophytes* ITS Type VIII strains from Germany formed their own clade, which is clearly discriminated from the other, above mentioned, *T. mentagrophytes* genotypes. Rooted with *T. quinckeanum*.

The phylogenetic tree based on sequencing of the TEF1-α gene revealed a 100% concordance of all 29 isolates belonging to genotype VIII of *T. mentagrophytes* (Figure 4b). Within the phylogenetic tree, all *T. mentagrophytes* isolates from this study formed their own clade, which was clearly differentiated from other *T. mentagrophytes* genotypes, and from *T. interdigitale*.

The origin of all reference strains, and clinical or animal isolates and their sequences used here for comparison, is presented in Table 2.

### 3.3. Antifungal Resistance Testing and Point Mutation Analysis

In vitro resistance testing revealed that 13 (45%) out of 29 strains were terbinafine-resistant with breakpoints $\geq 0.2$ µg/mL. The remaining 16 strains (55%) were terbinafine-sensitive (Table 1). Point mutation analysis revealed that among 13 resistant strains, 10 exhibited Phe$^{397}$Leu amino acid substitution of the SQLE, indicative of in vitro resistance to terbinafine. Two resistant strains showed combined Phe$^{397}$Leu and Ala$^{448}$Thr amino acid substitutions, and one strain a single Leu$^{393}$Phe amino acid substitution. Out of 16 terbinafine-sensitive strains, Ala$^{448}$Thr was detected in nine strains, one of which also showed a new Val$^{444}$Ile substitution. The remainder of the sensitive strains exhibited no substitution. Out of 13 strains tested for triazole sensitivity, nine proved to be sensitive to both itraconazole and voriconazole. Three strains revealed resistance to both triazoles, with one strain also showing resistance to terbinafine, while the remaining strain exhibited resistance to voriconazole but not itraconazole. The accession numbers of sequences of the SQLE gene of all 29 investigated *T. mentagrophytes* strains are deposited at the NCBI database (Table 1).

### 4. Discussion

#### 4.1. *Trichophyton mentagrophytes* Type VIII (India)

ITS genotype VIII of *T. mentagrophytes* seems to have grown rapidly in recent years to become the predominant dermatophyte in India [18]. In contrast, the previously predominant *T. rubrum* for decades was isolated with much less frequency [19]. A currently published epidemiological study of a total of 402 Indian patients with extensive dermatophytoses revealed culture growth of *T. mentagrophytes* in 289 (71.9%) of samples [15]. *T. rubrum* was cultivated from only 19 (4.7%) samples. It was possible to identify *T. interdigitale/T. mentagrophytes* complex in 235/265 (88.7%) of samples by PCR-ELISA. DNA sequencing enabled identification of *T. mentagrophytes* ITS Type VIII in 311 (77%) samples, unspecified species of *T. interdigitale/T. mentagrophytes* complex in 21 (5%), and *T. rubrum* in 19 (5%) samples.

It is interesting to note that *T. mentagrophytes* Type VIII was not initially found in India, but was previously isolated in Oman, Iran and also in Australia under a different species name, *T. interdigitale*, in accordance with the old taxonomy of dermatophytes that was valid only until 2016 [20–23]. Currently, the species found in India and several other countries globally is referred to as *T. mentagrophytes* Type VIII in the dermatomycological community [24]. *T. mentagrophytes* Type VIII is only one variety within the cluster of a large number of genotypes of the *T. mentagrophytes*/*T. interdigitale* complex [16,21,25–30] (Figure 5a–d). Therefore, it does not appear justifiable to attempt assigning this single genotype VIII of *T. mentagrophytes* to a brand new species, so hurriedly, on the basis of just two isolated terbinafine-resistance isolates of *T. mentagrophytes*, disregarding the plethora of literature on a
well-accepted taxonomy and nuances of this genotype, as has just happened in an isolated publication from Japan [31].

![Image](a) (b) (c) (d)  

**Figure 5.** 58-year old male originating from India suffering from tinea faciei due to *T. mentagrophytes* Type VIII. (a) Primary culture on slant agar tubes containing Sabouraud’s dextrose agar. Granular white, flat, fast-growing fungal colonies. (b) Yellow to brown stained reverse side of colonies on slant agar tubes. (c) Subculture of *T. mentagrophytes* Type VIII on Sabouraud’s dextrose agar petri dish agar. White, radiating and granular colonies with slightly yellowish stained centre of the colonies. (d) Single colony of *T. mentagrophytes* Type VIII on Sabouraud’s dextrose agar petri dish.

4.2. Spread of *T. mentagrophytes* Type VIII (India) to Other Parts of the World

Numerous reports and findings from our own laboratory suggest that the frequently terbinafine-resistant dermatophyte *T. mentagrophytes* Type VIII is becoming increasingly prevalent in countries other than Germany and beyond. *T. mentagrophytes* Type VIII has been isolated from skin scrapings of patients in Iraq, Switzerland, Cambodia, Finland, Estonia and Poland [32,33]. We have isolated only individual strains of *T. mentagrophytes* Type VIII since 2016, albeit initially under a different name of the species, owing to the classification at that time. There has been a significant increase in the frequency of detecting *T. mentagrophytes* Type VIII in the past two years in Germany and we regularly see a strain of *T. mentagrophytes* type VIII in routine diagnostics about every two to three weeks, which appears significant. The isolates of *T. mentagrophytes* Type VIII originate from all over Germany. Patients with dermatophytoses, due to the Indian genotype of *T. mentagrophytes*, have been identified in large cities like Berlin, Munich and Leipzig, as well as small towns and rural areas of Germany (Figure 6).
T. mentagrophytes with breakpoints < 0.2 µg/mL. The other 16 strains showed normal terbinafine susceptibility in vitro against terbinafine both in vitro and due to genetic point mutations in the SQLE gene. Some strains were also found to be partially resistant against itraconazole and voriconazole. Several single point mutations in the fungal SQLE gene, which encodes the target for terbinafine, have also been recorded in T. rubrum and T. mentagrophytes/T. interdigitale. These mutations have led to substitutions at one of the four amino acid positions Leu\(^{393}\), Phe\(^{397}\), Phe\(^{415}\) and His\(^{440}\), and have been associated with terbinafine resistance [12,34]. 71% of isolates of T. mentagrophytes Type VIII from India were found to be resistant to terbinafine [15]. The amino acid substitution Phe\(^{397}\) Leu in the squalene epoxidase of resistant T. mentagrophytes was found to be highly prevalent (91%) [15]. Two novel substitutions in resistant Trichophyton strains isolated in our currently published epidemiological study in India, Ser\(^{395}\)Pro and Ser\(^{443}\)Pro, were detected. In contrast, a missense substitution, Ala\(^{449}\)Thr, was found in terbinafine-sensitive and resistant isolates. Among the 29 strains isolated in Germany, 13 strains (45%) were terbinafine-resistant with breakpoints > 0.2 µg/mL. The other 16 strains showed normal terbinafine susceptibility in vitro against terbinafine with breakpoints < 0.2 µg/mL. It is not clear if all cases of terbinafine resistance occurred after long-term treatment with the drug, or if they had primary resistances. Some patients with resistance were pretreated by oral or topical terbinafine; unfortunately, because the biggest part of our patient’s data...
on pretreatment were not available. Indeed, however, transmission from affected people to family members who were definitely not pretreated, occurred.

It has been observed that despite in vitro susceptibility, there is often a poor clinical response to terbinafine, as seen in a significant number of patients reported herein. There are some indications of a lack of in vivo correlation of in vitro resistance in dermatophytosis [35]. On the other hand, it was demonstrated that the odds of achieving a cure with terbinafine MIC $< 1 \mu g/mL$ strains were 2.5 times the odds of achieving a cure with strains exhibiting MIC $\geq 1 \mu g/mL$, suggesting a good in vitro and in vivo correlation [36]. Further studies are recommended to understand this complex problem of the in vitro/in vivo discordance.

We demonstrated that in vitro resistance to triazoles cannot only be observed in $T$. mentagrophytes Type VIII isolated in India, but also in strains from patients residing in Germany. Remarkably, three out of four strains showing resistance to voriconazole were also resistant to itraconazole, strengthening evidence that these strains share a common mechanism of resistance against triazoles [15]. Triazole resistance was recently associated with the substitution Ala$^{448}$Thr in squalene epoxidase [15,30] and such a tendency could be observed in the current collection of strains. However, statistical significance could not be proven due to the relatively small sample size. Further investigations, especially on the role of SQLE double mutants on antifungal susceptibility [30], need to be carried out.

4.4. Treatment of Chronic Recalcitrant Dermatophytoses Due to $T$. mentagrophytes Type VIII

Patients described in this study represented the first reports on an infection due to a terbinafine-resistant $T$. mentagrophytes strain of the ITS genotype VIII from Germany and the Indian subcontinent. We aimed to highlight the recalcitrance to even long-term oral and topical treatment with terbinafine observed in our patients of tinea corporis and tinea cruris caused by $T$. mentagrophytes Type VIII. Unlike the scenario reported from India, the disease in German patients, seemed to respond to simple topical antifungal therapy other than terbinafine, as exemplified in the case of the baby originating from Bahrain with extensive tinea corporis [17]. It is interesting to note that the child showed significant improvement in the lesions after only one week of local treatment with miconazole and ciclopirox olamine, finally leading to resolution of all lesions with the same topical therapy for a total of four weeks. However, the lesions recurred after reaching Bahrain, where they stopped applying the topical antifungal agents. This case scenario has been experienced by several other patients with chronic, refractory dermatophytoses caused by $T$. mentagrophytes Type VIII. Treatment included topical ciclopirox olamine plus miconazole, sertaconazole or luliconazole with patients reporting more benefit with creams containing newer topical azoles. Many such creams are, unfortunately, not approved for use in Germany and other countries in Europe [37]. It seems appropriate to treat such cases with an oral antifungal agent like itraconazole in its adult dose of 100 mg twice daily after a meal for at least four to eight weeks. Some dermatologists recommend higher doses of the drug for a longer duration in widespread disease in patients who have abused topical steroid antifungal combinations for long periods.

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