

## LABORATORY DIAGNOSIS OF THE CAUSATIVE DERMATOPHYTES OF TINEA CAPITIS

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### ABSTRACT

Most laboratories do not perform mycologic examinations for diagnosis of tinea capitis because laboratory practitioners still believe that mycological procedures are too difficult to do and that the fungi are too infectious to handle require specialized mycologist. This discussion presents conventional and modern methods suitable for use in laboratories of all sizes especially laboratories with limited resources. It is hoped that laboratories will take the initiative in offering diagnostic mycology services for Tinea capitis routinely to be both a rewarding experience to those who choose to become involved and provides an accurate diagnostic services for patients care and

professional support and response to the requested tests by clinicians.

**KEYWORDS:** Tinea capitis, dermatophytes, laboratory diagnosis, KOH preparation, staining methods, culture method, Molecular assays.

### INTRODUCTION

Tinea capitis is a fungal infection of skin and hair of the skull commonly seen in children especially school children. It characterized by pruritic, scaling areas of hair loss and scalp lesions of two types, dry lesions that caused by anthropophilic fungi and wet lesions that caused by zoophilic fungi. The causative pathogens belong predominantly to two genera: *Trichophyton* and *Microsporum*.<sup>[1,9]</sup> Dermatophytes infections have a worldwide distribution but are endemic to tropical regions.<sup>[2]</sup> Laboratory methods include, Microscopy provides the most rapid means of diagnosis, Positive microscopy (when the hairs or scales are seen to be invaded by spores or hyphae) confirms the diagnosis and allows treatment to commence at once. Another method is inoculating samples obtained with a sterile single use brush on to a suitable culture medium, Culture is more specific than microscopy; results may be positive

even when microscopy is negative, but may take up to 4 weeks to become available.<sup>[3]</sup> Additionally, in recent years several molecular biology-based methods for identification of dermatophytes directly from clinical samples have been published.

### CLINICAL MANIFESTATIONS OF TINEA CAPITIS

The clinical manifestations of tinea capitis depending on the etiologic agent and the type of hair parasitization, tinea capitis are divided into four main forms: Microsporiasis, Trichophytosis, favus of the scalp and kerion.<sup>[4]</sup>

#### Microsporiasis

Microsporiasis defined as an infection caused by *Microsporum* species which occur mostly in school going children and families through which transmitted from one to another in addition to family pets.<sup>[5]</sup> The common *Microsporum* species causing tinea capitis are *Microsporum audouinii*, *Microsporum canis* and *Microsporum ferrugineum*. *Microsporum audouinii* an anthropophilic fungus causing non-inflammatory infections of the scalp and skin. *Microsporum audouinii*, which once caused large epidemics in Europe.<sup>[6]</sup> Invaded hairs show an ectothrix infection and usually fluoresces a bright greenish-yellow under Wood's ultra-violet light.<sup>[7]</sup> *Microsporum canis* is a zoophilic dermatophyte which is a frequent cause of ringworm in humans, especially in the children. *Microsporum canis* invades hair, skin and rarely nails. Cats and dogs are the main sources of infection. Invaded hairs show an ectothrix infection and fluoresces a bright greenish-yellow under Wood's ultra-violet light.<sup>[8]</sup> *Microsporum ferrugineum* is an anthropophilic fungus causing epidemic juvenile tinea capitis in humans. The clinical features are similar to those of infections caused by *M. audouinii*. Invaded hairs show an ectothrix infection and fluoresces a greenish-yellow under Wood's ultra-violet light.<sup>[9]</sup>

#### Differential diagnosis of Microsporiasis

Includes, alopecia areata, may show erythema, and although of itself, it is not a scaly condition. Seborrhoeic dermatitis is usually more diffuse than tinea capitis. Discoid lupus erythematosus and lichen planus.<sup>[10]</sup>

#### Trichophytosis

Term trichophytosis applies to all infections of fungi caused by the genus *Trichophyton*. Trichophytosis is most common among children especially in countries with climates that are more temperate. Clinical features of Trichophytosis consist of a large number of small grey

patches; and mild erythema. The patches are characterized by partial hair loss and visible as small stub. The lesions cover with grey scale and may become confluent. As in Microsporiasis healing can occur after puberty. Major causative agents of Trichophytosis are *Trichophyton tonsurans* and *Trichophyton violaceum*. *Trichophyton tonsurans* an anthropophilic cause black-dot ringworm, inflammatory krion form and chronic non-inflammatory finely scaling lesions of skin, nails and scalp. *Trichophyton tonsurans* is common cause of tinea capitis in the Latin America and in the African-American population. Invaded hairs show an endothrix infection and do not fluoresce under Wood's ultra-violet light.<sup>[12]</sup> *Trichophyton violaceum* an anthropophilic fungus causing inflammatory or chronic non-inflammatory finely scaling lesions of skin, nails, beard and scalp, producing the so-called black-dot tinea capitis. Invaded hairs show an endothrix infection and do not fluoresce under Wood's ultra-violet light.<sup>[13]</sup>

### Differential diagnosis of Trichophytosis

As mentioned under Microsporiasis includes, Alopecia areata, Seborrhoeic Dermatitis, dandruff and psoriasis.<sup>[10]</sup>

### Favus

The favic type of infection is caused by the anthropophilic dermatophyte *Trichophyton schoenleinii*. favus is a chronic and scarring form of tinea capitis characterized by saucer-shaped crusted lesions or scutula and permanent hair loss. Invaded hairs remain intact and fluoresces a pale greenish yellow under Wood's ultra-violet light. Clinical features of favus begin as the patches increase in size and irreversible hair loss occurs in the center region. A salient feature is that hair is not broken off. Impetiginous form of favus is characterized by moist crusts with accumulation of pus. There is no tendency of healing after puberty, so if children not treated well it will be chronic and remaining till adult and may lead to permanent alopecia.<sup>[14]</sup>

### Kerion

The krion type of infection is violent reaction results from infection with an animal dermatophyte such as *Trichophyton mentagrophytes* var. *mentagrophytes* and *Trichophyton verrucosum*. Krion infection characterized by the inflammation spread in more than one part of skull with pus and loss of hair. Usually occurs in all ages.<sup>[15]</sup> The causative agents of kerion is *Trichophyton mentagrophytes* var. *Mentagrophytes*<sup>[16]</sup> a zoophilic form of *T. mentagrophytes* which is characterized with a wide range of animal hosts including mice, guinea pigs, kangaroos, cats, horses, sheep and rabbits. Often produces inflammatory skin or

scalp lesions in humans, particularly in rural workers. Kerion of the scalp and beard may occur. Invaded hairs show an ectothrix infection but do not fluoresce under Wood's ultra-violet light.<sup>[17]</sup> *Trichophyton verrucosum* which is a zoophilic fungus causing ringworm in cattle. Infections in humans result from direct contact with cattle or infected fomites and are usually highly inflammatory involving the scalp, beard or exposed areas of the body. Invaded hairs show an ectothrix infection and fluorescence under Wood's ultra-violet light has been noted in cattle but not in humans. All strains require thiamine for growth and many strains also require inositol. Unlike other dermatophytes growth is enhanced at 37°C.<sup>[18]</sup> Clinical features of Kerion begin as erythematous annular patch which gradually elevates itself above surrounding skin. Is not restricted to scalp and may extend to beard and with well response to treatment and no hair loss. If not treated the disease will persist for several weeks or months, then the symptoms will gradually diminish but an atrophic scar may remain after healing while the sustained hair loss is not be fully replaced.<sup>[19]</sup>

#### PREVENTION OF TINEA CAPITIS

Tinea capitis may prevented by keeping the patients at home for few days to prevent others uninfected persons. If a zoophilic fungus is an agent of infection as *M. Canis* infected animal must be treated to prevent reinfection of the carriers. *M. canis* and *T. verrucosum* infections of cats and dogs can often be detected with Wood's light.<sup>[11]</sup>

#### LABORATORY DIAGNOSIS OF TINEA CAPITIS

Laboratory diagnosis is highly recommended to diagnosis and confirms that the relevant organism is identified as different fungal species may require specific treatment approaches.

#### Collection of specimens

Taking samples starts with cleaning the affected area with 70% v/v ethanol then collection of skin scales, crusts and hair pieces on a clean piece of paper about 5 cm square using dark coloured paper so that the specimen is easy to see.<sup>[20,21]</sup> If lesions are inflamed and painful, using the sterile distilled water is recommended.<sup>[21]</sup>

**Skin scales:** Collect by scraping the surface of the margin of the lesion using a sterile blunt scalpel.<sup>[20,21]</sup>

**Crusts:** Collect by removing part of the crust nearest to healthy skin using sterile scissors and tweezers.<sup>[20]</sup>

**Hairs:** Collect by removing dull broken hairs from the margin of the lesion using sterile tweezers or scraping the scalp with a blunt scalpel.<sup>[20]</sup> Hair for black-dot endothrix infections should be dug up from the follicle with the point of a sterile scalpel. In favus, the scutulum at the mouth of the hair follicle is suitable for culture and microscopic examination.<sup>[21]</sup> Approximately 5 to 12 hair stumps should be collected.<sup>[21,22]</sup> Alternately, sterile disposable brushes may be used to collect hairs. This method is especially suitable to collect hairs from small children.<sup>[21]</sup>

### **Transporting fungal specimens to the laboratory**

Tinea capitis specimens are best transported in paper packages rather than screw-cap containers to reduce humidity and the multiplication of bacteria. Spores of ringworm fungi resist drying and remain viable for several months when stored in paper. The paper should be folded to enclose the specimen and use a paper clip to close it.<sup>[23]</sup> Then specimens should be labeled with the patient's name and number, source of material and the date.

## **DIRECT MICROSCOPICAL METHODS**

### **Potassium hydroxide (KOH) preparation**

Fungi are larger than bacteria, so they are easy to see by direct microscopy in skin or hair specimens.<sup>[20]</sup> Provided specimens are first softened and cleared with a strong alkali such as (20% w/v) potassium hydroxide (KOH).<sup>[20,24,25]</sup> The purpose of the alkali is to digest the keratin surrounding the fungi so that the hyphae and conidia (spores) can be seen.<sup>[20,27]</sup> KOH test is highly sensitive, specific and recommended for diagnosis dermatophytes infections due to time-consuming of fungal culture as well as additional costs. Moreover, treatment can be started in most cases if clinical signs are correlated to KOH test positive results.<sup>[26,27]</sup> Addition of blue-black fountain pen ink to KOH preparation is preferred by some laboratories, but the ink is not specific for fungi and can stain cells and other components of the skin.<sup>[20,27,33]</sup>

KOH test procedure starts by placing a drop of KOH solution on a microscopic slide, then adding of the specimen (small pieces) to the drop of KOH, and cover with a cover glass. Place the slide in a Petri dish, or other container with a lid, together with a damp piece of filter paper or cotton wool to prevent the preparation from drying out. To assist clearing, hairs should not be more than 5 mm long, and skin scales and crusts should not be more than 2 mm across. Hairs clear within 5–10 minutes. Skin scales and crusts usually take 20–30 minutes. Clearing can be hastened by gently heating the preparation over the flame of a spirit lamp or

pilot flame of a Bunsen burner, Taking into account preventing dryness of the preparation and splatter of the of the corrosive KOH solution. As soon as the specimen has cleared, examine it microscopically using the 10x and 40x.<sup>[28]</sup>

### **Reporting KOH preparation - skin scales and crusts**

KOH preparation slides should be scanned for presence of branching septate hyphae with angular or spherical arthroconidia (arthrospores), usually in chains.<sup>[29,30]</sup>

### **Reporting KOH preparation - infected hair**

KOH preparation slides should be scanned for presence of arthroconidia and hyphae. arthroconidia may be arranged along the length of the hair in chains or they may be found in masses around the hair. Endothrix infection: Look for infection in the hair substance. A special type of endothrix infection is shown by *T. schoenleinii*, the dermatophyte that causes favus. *T. schoenleinii* hyphae do not break up into arthroconidia but eventually die leaving characteristic air spaces outside of the hair (ectothrix) or within it (endothrix).<sup>[31,32]</sup> Ectothrix infection: The arthroconidia may be arranged along the length of the hair in chains or they may be found in masses around the hair, if none of the fungal structures is found in the entire smear, report is dispatched as “*NO FUNGAL ELEMENTS SEEN*”.<sup>[32]</sup>

### **The Parker-KOH stain**

The Parker-KOH stain is combination of Parker's ink and KOH solution potassium hydroxide. Parker's ink stains the fungal wall blue and is thus easy to recognize. The Parker-KOH stain containing 1 part Parker blue-black ink and 1 part 20% KOH.<sup>[35,36]</sup>

### **Lactophenol cotton blue wet mount**

Lactophenol cotton blue (LCPB) mount use to visualize microscopic fungal morphology by staining the cell walls by blue color. A one drop of LCPB is added to specimen, a coverslide is applied. And the preparation examined microscopically.<sup>[37]</sup>

### **Chicago sky blue (CSB) stain**

The CSB stain is a new contrast stain that has shown promising results as a rapid and reliable diagnostic method for dermatophytoses. The CSB stain performance is greatly enhanced by using KOH as a clearing agent. Preparation of 1% CSB –10% KOH stain by adding 1 g of CSB powder into 100 ml of 10% KOH, staining procedure is by mixing a drop of the stain with the specimen on a clean microscope slide, and keeping smear in a covered plastic

container lined by moist paper towel to prevent drying for about 20 minutes at room temperature. Then cover smear with a coverslip and gently press to remove air bubbles then examine smear microscopically using magnification x10 and x40. Positive smear will show fungal elements stained blue against purplish background.<sup>[34, 35]</sup>

#### **Calcofluor white stain**

Calcofluor white is a fluorescent stain that is mixed with KOH (1 drop KOH: 1 drop Calcofluor white) in order to stain the chitin in the fungal cell wall, thus making fungal elements more easily visible against the background of host cellular material. However, as with KOH, the identity and viability of microorganisms cannot be determined. Calcofluor white binds to the beta 1-3 and beta 1-4 polysaccharides in cellulose and chitin and fluoresces when exposed to UV radiation from fluorescence microscope. Peak excitation and emission wavelengths for calcofluor white solution are 365 and 435 nm, respectively.<sup>[38]</sup>

#### **Periodic acid–Schiff stain**

The periodic acid–Schiff (PAS) stain is highly sensitive histopathological stain of fungal Polysaccharides. PAS is used for the diagnosis of tinea capitis to identify hyphae and spores within or around hair shafts and skin specimens. PAS is highly sensitive when coupled with fungal culture. The sample is sectioned and stained with PAS stain, which reacts with the aldehyde groups in the fungal cell walls to produce a magenta-colored fungi against light green background. In addition to high sensitivity, PAS staining results are available quickly, usually within 24 to 48 hours. However, as with KOH testing, the main disadvantages of PAS staining are that the identity of microorganisms cannot be confirmed and that viable and nonviable organisms are indistinguishable. Furthermore, the cost associated with PAS is often higher than KOH technique.<sup>[40]</sup>

#### **Gomori's methenamine silver stain**

Gomori's methenamine silver (GMS) stain is the most sensitive technique used to identify dermatophytes. The principles behind GMS staining are similar to PAS staining, GMS highlight the wall of the fungus and thus are useful for screening the sample. GMS can be combined with Hematoxylin and eosin in such a way that the fungus and the host reaction can be clearly observed. The fungal cell wall appears black or dark brown for all fungi with green surrounding tissue.<sup>[42]</sup>

## FUNGAL CULTURE-BASED METHOD

Fungal culture has been considered to be the 'gold standard' technique in the diagnosis and identification of dermatophytes. Culture procedure starts with plating clinical samples onto a properly selected general media such as potato dextrose agar with added antibiotics to inhibit overgrowth by bacterial contaminants and a selective media such as Sabouraud dextrose agar (SDA) base and the other in a Mycosel agar base, both to be supplemented with chloramphenicol (which inhibits bacteria) and cycloheximide (to inhibit saprophytic fungi). Incubate cultures aerobically at room temperature (25°C) for up to 4 weeks. Examine culture both macroscopically for positive results (color of the surface and reverse, topography, and texture) and microscopically (microconidia and macroconidia) for species identification. In the absence of any growth after 4 weeks, the culture will be considered negative. A significant advantage of using fungal culture is that it is able to identify the causative agent. False negatives can occur for a number of reasons, including insufficient specimens or improper sample collection due to too far removal of sample from the site of infection. In order to reduce the likelihood of obtaining false-negative results, the specimen should be collected as proximally to the infected area most likely to contain viable organisms as possible and least likely to contain contaminants. False-positive results may very rarely be caused by contamination from organisms present as transient flora.<sup>[39]</sup>

Performing antifungal susceptibility testing by probing dermatophytes colonies from mycosel agar cultures using the tip of a sterile Pasteur pipette to obtain a mixture of mycelium and conidia and mix in 1ml distilled water; suspend the mixture in sterile tubes and allow to sediment for 30 minutes; Dip swab into the inocula suspensions and streak evenly over the surface of Mueller-Hinton (MH) agar plates. Leave lids left ajar for 3 minutes in a laminar flow cabinet to allow for any excess surface moisture to be absorbed into the agar before applying the drug-impregnated disks. Many antifungal agents can be used as Clotrimazole (50 µg), Miconazole (10 µg), Fluconazole (25µg), and Griseofulvin (10 µg). Then incubate the plates at 25°C for 5 to 10 days. When growth took place, measures the size of the zones of inhibition around the disks.<sup>[41]</sup>

## MOLECULAR METHODS

In recent years, several diagnostic methods for identification of dermatophytes directly from clinical samples using molecular biology-based methods have been published. Different techniques have been used, such as PCR-reverse line blot (PCR-RLB)<sup>[47]</sup>, real-time PCR

using sets of species-specific primers and probes<sup>[43]</sup>, multiplex PCR with pan-dermatophyte<sup>[46]</sup> and PCR-ELISA test.<sup>[44]</sup> The genes most frequently used as the main targets for dermatophyte PCR are: the Chitin Synthase 1 (CHS1) gene, the ITS regions in the rDNA gene and the topoisomerase II gene. Recently, the fields of fungal characterizations by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) have been explored.<sup>[48]</sup>

### Real-time PCR assay

Kobylak *et al.*,<sup>[43]</sup> described a real-time PCR test for detection of dermatophytes using pan-dermatophyte primers and fungal DNA from pure culture. This involves DNA extraction procedure followed by real-time PCR. This method may be applied in routine diagnostic laboratories due to 100% specificity and selectivity as well as significant reduction of analysis turnaround time. PCR Procedure starts with DNA extraction by resuspending small fragment of mycelium in 100 µl of extraction buffer (60 mM NaHCO<sub>3</sub>, 250 mM KCl and 50 mM Tris, pH 9.5), followed by 10 min incubation at 95°C. Next, adding 100 µl of neutralization buffer (2% bovine serum albumin). Then Real-time PCR step by using specific primers to detect DNA fragment encoding chitin synthase 1, panDerm1 and panDerm2. PCR mixture consisted of 10 µl SybrA, 0.2 µl of each primer (panDerm1 and panDerm2) at 100 µM and 2 µl of DNA in a volume of 20 µl. Use the real-time PCR device the time-temperature profile for PCR is 5 min initial denaturation at 95°C, 40 cycles consist of: 15 sec denaturation at 95°C, 15 sec annealing at 55°C, acquisition of signal after 25 sec elongation at 72°C. Then melting curve analysis (15 sec at 95°C, 20 sec at 40°C at ramp of 4°C/s and 95°C for 20 sec at ramp of 0.1°C/s). The melting temperature of the generated PCR products in the range of 87 to 92°C indicates the presence of dermatophytes DNA in the sample.<sup>[43]</sup>

### Polymerase chain reaction PCR-ELISA assay

Recent study by Beifuss *et al.*,<sup>[44]</sup> described an effective combination of PCR assay and enzyme-linked immunosorbent assay (PCR-ELISA) for rapid detection of dermatophyte species directly from clinical specimens within 24 hour. Isolated genomic DNA specimens are amplified with species-specific primers then PCR products subsequently detected using biotin-labeled probes. This method was used for detection of the five common dermatophytes, *T. rubrum*, *T. interdigitale*, *T. violaceum*, *M. canis*, and *E. floccosum*, from clinical specimens. Genomic DNA can be isolated from skin samples from patients with suspected dermatophyte infections and then amplified with species specific primers. After

direct DNA extraction from clinical specimens and PCR testing to identify clinical isolates then the PCR products to be hybridise with 50 -biotinylated species-specific oligonucleotide probes. The hybridised DNA then transferred into microwells coated with streptavidin, and the revealing amplified DNA by using anti-digoxigenin-horseradish peroxidase antibodies and peroxidase substrate. ELISA-based detection was found to be ten times more sensitive than conventional gel electrophoresis. The experiment shows coupled PCR-ELISA could provide a rapid, reproducible and sensitive tool for detection and discrimination of five major dermatophytes at species level, independent of morphological and biochemical characteristics.<sup>[44]</sup>

### **Multiplex PCR assay**

Multiplex PCR provides a powerful tool for simultaneous detection and discrimination of multiple pathogens or different subtypes of a causative agent from humans, animals, and plants in a single reaction.<sup>[45]</sup> Study by L. Mehlig *et al.*,<sup>[46]</sup> using this technique, DNA was extracted from clinical specimens, then Polymerase chain reaction was performed using kit consists of all reagents to perform two separate multiplex PCRs firstly, Primer mix 1 contains specific PCR primer pairs for *E. floccosum*, *M. canis*, *Microsporium gypseum*, *Trichosporon cutaneum*, *S. brevicaulis*, *Aspergillus spp.*, *Candida spp.* and an unrelated internal amplification control. Another Primer mix 2 supplies specific PCR primer pairs for the amplification of *T. rubrum*, *T. interdigitale*, and *Trichophyton spp.* The study concluded that from DNA isolation to diagnosis the multiparameter diagnostic kit gives rise to a 1-day workflow enables fast clarification of disease aetiology.<sup>[46]</sup>

### **PCR-reverse line blot assay**

In a recent study, Bergmans *et al.*,<sup>[47]</sup> developed and successfully used a PCR-reverse line blot (PCR-RLB) for rapid detection and identification of nine dermatophyte species in nail, skin, and hair samples. The developed method was based on Internal transcribed spacer 1 (ITS1) sequences using genus and species-specific probes. Membranes containing immobilized oligonucleotide probes were exposed to denatured PCR products and allowed to hybridize for 30 minutes, then subjected to stringency washes and detection using streptavidin-peroxidase and chemiluminescence. The investigators reported a positive PCR-RLB reaction in 93.6% of 172 culture-positive and microscopy- positive samples.<sup>[47]</sup>

### MALDI-TOF assay

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry has emerged as a reliable technique to identify molds involved in human diseases, the ability to analyze large biological molecules by mass spectrometry is made possible by the application of soft ionization techniques that generate a spectrum of components. In a MALDI-TOF analysis, a saturated solution of an organic compound or matrix is added to a portion of a fungal colony, and the mixture is then applied to a metal plate. Upon drying, the crystallized mixture is subsequently irradiated using a laser beam to force sublimation into a gas phase, followed by ionization of the fungal sample. Ionized proteins within the sample are analyzed by a mass spectrometer analyzer to produce a spectrum of mass-to-charge(m/z) ratios. These ratios measure how quickly charged ions from the fungal sample move through the time of flight (TOF) tube. Once spectra are generated, comparison of the m/z ratios to a reference database leads to fungal identification. Protein compositions differ between fungal species, which allows for discrimination between closely related organisms. Clearly, MALDI-TOF will prove an invaluable methodology for rapid identification of dermatophyte isolates once commercial databases become more robust. However, current limitation of this method is the necessity for isolating pure dermatophytes colonies from clinical samples before MALDI-TOF assay can be attempted.<sup>[48]</sup>

### CONCLUSIONS

The present review of laboratory diagnosis of the causative dermatophytes of tinea capitis, conclude that the available laboratory techniques support the role of laboratory in rapid and accurate diagnosis of various dermatophytes infections especially causative agents of tinea capitis. These methods may be implemented in wide range of laboratories size, which also allow laboratory personals to select appropriate assay to be indoor laboratory service then, accurate and rapid diagnostic report available for patients and clinicians.

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