

British Journal of Dermatology, Volume 155, Issue 6, pp1209-1216, December 2006

***Trichophyton* species: use of volatile fingerprints for rapid identification and discrimination**

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Key words: *Trichophyton* species, early detection, electronic nose, volatile fingerprints

Conflict of interest: None

## Summary

*Background:* Fungal infection of the skin is a common clinical problem, and laboratory confirmation of the diagnosis is important to ensure appropriate treatment. The identification of the species of fungus is also important, because different fungal species have different modes of transmission, and this may be of importance both in preventing re-infection or in avoidance of infection of others.

*Objective:* This study examined the potential of using volatile production patterns for the detection and discrimination between four *Trichophyton* species (*T. mentagrophytes*, *T. rubrum*, *T. verrucosum* and *T. violaceum*) in vitro on solid media and in broth culture.

*Methods:* Two different sensor array systems (conducting polymer and metal oxide sensors) were examined for comparing the qualitative volatile fingerprints produced by these species over periods of 24-120 hrs in the headspace. The relative sensitivity of detection of two of the species (*T. mentagrophytes*, *T. rubrum*) was determined for log<sub>1</sub> to log<sub>7</sub> inoculum levels over the same time period.

*Results:* The conducting polymer based system was unable to differentiate between species based on volatile fingerprints over the experimental period. However, metal oxide-based sensor arrays were found to be able to differentiate between the four species within 96 hrs of growth using PCA analysis which accounted for approximately 93% of the data in PC1 and 2 based on the qualitative volatile production patterns. This differentiation was confirmed by the Cluster analysis of the data using Euclidean distance and Ward's linkage. Studies of the sensitivity of detection showed that for *T. mentagrophytes* and *T. rubrum* it was possible to differentiate between log<sub>3</sub>, log<sub>5</sub> and log<sub>7</sub> inoculum levels within 96 hrs.

*Conclusions:* This is the first detailed study of the use of qualitative volatile fingerprints for identification and discrimination of dermatophytes. This approach could have potential for rapid identification of patient samples reducing significantly the time to treatment.

## Introduction

Dermatophytes are one of the most common human fungal infectious diseases in the world and the leading cause of hair, nail and skin infections in humans. Infection is usually restricted to the cutaneous layers of the skin where dead tissue layers prevail<sup>1</sup>. The *Trichophyton* group of species are particularly important anthrophilic species. Isolation and rapid recognition of such dermatophytes to species level is important to enable appropriate remedial drug treatments to be provided. At present this requires samples to be plated on agar-based media to isolate the dominant species present and diagnose whether the relevant species is responsible for the infection. This often requires 7-10 days for fast growing species and up to three weeks for slow growing species, e.g. *T. violaceum*. Molecular diagnostics approaches have also shown promise, although in some cases DNA sequences have been found to be similar to other non-dermatophyte species<sup>2,3</sup>. These are neither very cost effective nor suitable for routine clinical testing.

Previous studies have demonstrated that different yeasts and filamentous fungal species produce specific volatile compounds, including alcohols (e.g. butanols, propanols, hexanols and octenols), ketones (e.g. propan-, butan-, penta-, and heptan-2-ones), aldehydes (e.g. hexanal, 2- and 3-methyl-butan-1-als), and aromatics (e.g. benzaldehyde, benzene based compounds) and methyl, butyl and ethyl esters when analysed using GC-MS<sup>4</sup>. Sensor arrays have been developed which when exposed to the different volatile groups result in a change in their physical properties such as mass, conductivity or capacitance which can be measured. It is essential that individual sensors have varying sensitivities to the same substance so that different odours have a distinct volatile response pattern across the sensors enabling an unknown odour to be identified<sup>5</sup>. Thus by assembling appropriate sensor arrays it is possible to obtain a volatile production fingerprint. By using statistical techniques the data can be

analysed to try and discriminate between these volatile fingerprints. This has resulted in the development of electronic noses which consist of three basic building blocks: a sensor array, signal conversion system, and software analysis resulting in an output/result i.e. pattern recognition system. Care is needed to prevent the sensors becoming saturated and thus affecting responses to volatiles. These systems are thus ideally placed to be used for early and rapid detection of microbial activity in environmental, food and medical applications<sup>6</sup>. There are different sensor array systems which have been used and described in detail elsewhere<sup>5,6</sup>. The two used in the current study: conducting polymer based systems work at ambient temperatures, while metal oxide and metal ion-based sensors worked effectively at elevated temperatures (200-500°C).

While a significant body of research exists on the application of volatile fingerprints for the detection of fungi in food matrices less attention has been given to medical mycological applications<sup>6,8-11</sup>. Recent studies have demonstrated that it was possible to differentiate between bacteria responsible for UTI and also the analyses of sputum samples for the detection of TB<sup>11-12</sup>. However, no detailed studies have been conducted in relation to filamentous fungal infections. With regard to dermatophytes the ability to discriminate between types of infecting species within a few days of isolation would be a major advantage.

The objectives of this study were to (a) evaluate two electronic nose-based systems based on conducting polymer and metal oxide sensor arrays for detecting volatile production patterns produced by *Trichophyton* species, (b) using the best system to determine the optimum time at which effective discrimination between four different *Trichophyton* species could be done over periods of 24-120 hrs growth on different solid agar and in liquid broth cultures and (c) determine the sensitivity threshold for detection of two individual species, *T. mentagrophytes* and *T. rubrum* when present at initial concentrations of log<sub>1</sub>-log<sub>7</sub> CFUs ml<sup>-1</sup>.

## **Materials and Methods**

### **Fungal species**

The four species used in this study were *Trichophyton mentagrophytes* (stat. conid. of *Arthroderma vanbreuseghemii*, No. 224); *T. rubrum* (No.115 and strain D12); *T. verrucosum* (No. 685); and *T. violaceum* (No. 677). All were human isolates and obtained from the National Collection of Pathogenic Fungi, Bristol, U.K., except strain D12 which was kindly provided by the University of Oxford.

### **Cultivation media**

For in vitro studies the cultures were grown on two types of solid media:

Sabouraud Brain Heart Infusion (SABHI) Agar was prepared by mixing Brain Heart Infusion (BHI) agar (Oxoid) and supplementing with the required amount of Glucose (Acros Chemicals) with the addition of a small amount of the antibiotic, Chloramphenicol (Sigma) [47g l<sup>-1</sup> BHI + 38 g l<sup>-1</sup> Glucose]. The second agar-based medium was Sabouraud Dextrose Agar (SDA) which was prepared in house by mixing 10g l<sup>-1</sup> Mycological peptone (Amersham), 40 g l<sup>-1</sup> Glucose (Acros Chemicals) and 15 g l<sup>-1</sup> Agar technical no. 3 (Oxoid). A small amount of antibiotic, Chloramphenicol (Sigma) was also added. The liquid broth medium was Sabouraud Dextrose as prepared for SDA above, without the addition of the agar.

### **Discrimination between species**

At least 25 replicate agar plates were inoculated with 0.25 ml of a 10<sup>6</sup> spore ml suspension of each of the four *Trichophyton* species and spread plate on the agar surface. The plates were incubated at 25°C for 24-120 hrs and 5 replicate plates destructively sampled. Four 2 cm diameter discs of the cultures were placed into 25 ml vials left for 1 hr to equilibrate and then the headspace was analysed using the two sensor array systems. Studies were carried out with

each of the solid and liquid media and repeated at least twice. Initial studies suggested that at >120 hrs significant visible growth occurred and the amount of volatiles produced by all species resulted in saturation of the sensor arrays and thus less separation between fungal treatments.

### **Sensitivity of detection**

Studies were conducted with *T. mentagrophytes* and *T. rubrum* using both the solid agar media and liquid broth medium. Spore suspensions were made from 10-15 day old cultures and the initial concentration determined using a haemocytometer. The stock solution was diluted appropriately to obtain treatments of  $10^1$ ,  $10^3$ ,  $10^5$  and  $10^7$  CFUs ml<sup>-1</sup>. Treatment media were spore lawn inoculated and 25 replicates of each incubated at 25°C for up to 120 hrs as before. Studies were also carried out in the liquid SD broth medium in shaken cultures.

In all cases four 2 cm diameter agar discs from the solid media were placed in the sample tubes which were sealed with a septa and screw top lid. The samples were allowed to equilibrate for 1 hr before measurement of the volatile production profiles.

### **Electronic sensor array systems**

*Conducting polymer-based system:* Single replicate 9 cm diameter Petri plate cultures were placed in sampling bags (500 ml capacity), with the lid carefully removed, filled with filter-sterilized air and sealed. The bags were incubated for 1 h at 25°C, to equilibrate the headspace. The headspace from each bag was subsequently sampled through an air-filter system, which consisted of a bio-filter (0.45 µm, PTFE Whatman, Hepa-Vent) and an activated carbon filter (Whatman), to ensure clean airflow. An electronic nose system (BH114, Bloodhound Sensors, UK) that uses an array of 14 conducting polymer sensors was used in this study. Samples

were analysed randomly, including the controls. The data was collected and analysed by the e-nose software package system<sup>5</sup>.

*Metal oxide/ion sensor array system (Applied Sensors):* Samples were placed in the NST 3220 Lab Emission Analyser carousel. This system has 10 MOSFET sensors, 12 MOS sensors, a CO<sub>2</sub> and Humidity sensor. It employs an automated robotic needle to draw sample headspace from a sealed container sampling vial, and the sample exposed to the sensor array. Data were analyzed using principal component analysis (PCA) and cluster analysis (CA).

### **Data analysis**

The data collected was analysed by in-built software packages in the electronic nose systems and Statistica 7. For the Bloodhound, normalised data for divergence, a sensor parameter indicating maximum step response, was analysed using XLStat (a Microsoft Excel add-in). Multivariate statistics involving Principal Component Analysis (PCA) and hierarchical Cluster Analysis (CA) were applied to the obtained sensor responses.

Principal Component Analysis (PCA): This is an effective unsupervised and linear pattern recognition technique which helps identify general relationships within the data. It reduces the dimensionality of the data by preserving maximum information from the original data set (seeks a direction in space which captures maximal variance) with minimum number of variables. The new variables (principal components, PC) are derived such that the first PC accounts for as much of the variance as possible in the data, with each subsequent PC arranged in decreasing order of variance. It thus helps in summarising the data with little loss of information. The relationship between the samples can be visualised by means of a score plot.

Cluster Analysis (CA): This is another unsupervised, linear pattern recognition technique that enables one to establish/determine the relationships between samples and sample groups in a graphical way. Initially, the similarities between the samples are determined by measuring the distances between them, two at a time using the various distance measures (for e.g. Euclidean distance). Samples are said to be similar when the distance between them is short. Then clusters with the smallest distance are aggregated together by linkage algorithms (for e.g. Ward's method) to form larger clusters, the result of which is represented by means of a dendrogram i.e. tree-diagram<sup>13</sup>. The axis on the dendrogram denotes the linkage distance.

## **Results**

### **Discrimination between *Trichophyton* species**

Experiments were initially conducted with all four species grown on agar-based media and the headspace of up to 5 replicates per treatment were examined using both the conducting polymer and metal oxide/ion based sensor array for discrimination. The replication of the treatments was examined and found to be very consistent. Figure 1 compares the sensor array responses for the metal oxide/ion based system by comparing responses of blanks with a treatment species. There are clear differences in volatile fingerprints between treatments and the variation between replicates was relative small and consistent. The relative respiration of the treatments also increased with time, over the incubation period. Figure 2 shows that over the experimental time period (24 to 120 hrs) the respiration of each species was slightly different and generally increased with time. This period was chosen because it represents early microscopic and visible growth, when identification is difficult.

Figure 3 shows the PCA using the conducting polymer sensor array. This produced very little discrimination between the species examined. However, much better results were obtained with the alternative sensor array system. Within 72 hrs *T. mentagrophytes* and *T. rubrum* was differentiated from the other species based on the PCA analyses (highlighted by the author) (Figure 4). Figure 5 shows that after 96 hrs it was possible to separate all four species based on their volatile fingerprints (highlighted by the author). This accounted for approx 97 % of the data. Data after 24-48 hrs was found to be too early for adequate volatile production to enable discrimination between the species.

This was confirmed when the Cluster analysis (using the Euclidean distance measure and Ward's linkage) were examined between the different replicates and treatments. This showed that the blanks and all four species were clustered independently (Figure 6). Similar results were obtained in both agar-based and in the liquid broth media.

### **Threshold detection limits**

Studies were conducted with *T. mentagrophytes* and *T. rubrum* to identify the concentrations of spores at which volatile fingerprints could be used for detection. Within 72 hrs it was possible to discriminate between the log5 and log7 treatments. Figure 7 shows that after 96 hrs growth it was possible to differentiate between log3, log5 and log7 initial concentration using PCA 1 and 2 for *T. mentagrophytes* (highlighted by the author). This accounted for >99% of the data. However, log1 and blanks could not be effectively differentiated. This was confirmed by examining the Euclidean distances between the samples. The replicates were clustered separately for the log3-log7 treatments, with a mixed grouping of the blanks and log1 initial concentrations (Figure 8). Similar results were obtained for *T. rubrum* with a sensitivity of detection after 96 hrs of log3 initial concentration (data not shown).

## Discussion

This is the first study where qualitative volatile production patterns have been used to try and discriminate between growth of different *Trichophyton* species. This suggests that the responses of the metal oxide/ion sensor array systems were more sensitive to the volatiles produced by this group of filamentous fungi than the conducting polymer based system. This may be because the volatiles generated in the headspace by *Trichophyton* species are at very low concentrations initially or that the type of volatiles cause very small changes in the resistance of the conducting polymer sensor array resulting poor discrimination between treatments.

The use of qualitative volatile fingerprints for differentiating between the four species required at least 96 hrs growth, regardless of medium used. After 24hrs the amount of volatiles produced is too low to enable any discrimination between species. However, discrimination of the two faster growing species, *T. mentagrophytes* and *T. rubrum*, could be achieved earlier (48-72 hrs). Previous studies with filamentous fungi have shown that on food-based matrices it was possible to discriminate between *Aspergillus* and *Penicillium* species within about 48 hrs of growth, much earlier than other techniques such as enzyme assays and traditional plate counts, which required at least 72 hrs<sup>10</sup>. In these studies, no visible growth was present after 48 hrs. More recent studies to discriminate between a bacterium, yeast and filamentous fungal species showed that discrimination between the species was possible using the qualitative volatiles production patterns after 72 hrs. In these studies differences in some key volatiles such as 2-butanone, butanol, pentanol, methyl benzene and 1-hexanol were correlated with this discrimination using GC-MS<sup>5</sup>.

The threshold level of detection of an individual microbial species is important knowledge to enable the potential application of the technique for early and rapid detection of a pathogen or contaminant. This study suggests that for the two most common *Trichophyton*

species (*T. mentagrophytes* and *T. rubrum*) an initial concentration of about log3-4 spores can be discriminated. Lower initial concentrations (log1-2) produce very low levels of volatiles in the headspace making discrimination very difficult. However, this is comparable with detection limits of bacteria in water <sup>14</sup>, micro-organisms in milk <sup>9</sup> and for detection of microbial contaminants in other food matrices<sup>15</sup>.

This paper has described a potential new and novel method for the rapid identification of dermatophytes based on growth from microconidial cultures. Of course, the ability to directly examine skin scrapings or cultures from skin which may contain arthrospores may be more challenging. However, provided enough background data is acquired a library of information can be obtained for valid comparisons to be made and a diagnostic decision obtained. This would ensure that more rapid appropriate treatments can be made. The identification of the species of fungus is also important, because different fungal species have different modes of transmission, and this may be of importance, both in preventing re-infection or in avoidance of infection of others. For example, in a case of tinea corporis, the presence of *Trichophyton rubrum* should lead to investigation for other sites of infection, such as associated tinea pedis or tinea unguium, whilst the presence of *Microsporum canis* would suggest contact with a cat or dog.

Conventionally the presence of a dermatophyte may be confirmed by direct microscopy of skin scrapings in a potassium hydroxide preparation, and the species may be subsequently identified by the appearance on culture using Sabouraud's medium (or similar). Microscopy has the disadvantage that it requires time and a skilled physician or technician, commodities which are not universally available in a busy clinic; false negative results may occur through sampling error. Culture is slow, taking up to three weeks, and this may delay initiation of treatment. This time delay also creates difficulties when one is monitoring response to treatment (i.e. taking further specimens to confirm clearance) because the results

may be some weeks behind the clinical scenario. The fact that culture does take such a long time is also a reason why in practice it is not routine to undertake sensitivity testing in the way that is routine in bacteriology laboratories.

In summary, this study has shown a potential method for the rapid identification of dermatophytes which allows definition of different species with results available in 96 hours, and which may be capable of identifying the presence of fungi in very small quantities. If the technique could be applied in clinical practice it would present significant advantages to the clinician, in terms of early laboratory confirmation of diagnosis, and might even make it possible for sensitivity testing to be routine, thereby optimising treatment regimes. We propose to undertake a formal study of this technique in a clinical context in the near future.

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## Figure legends

Figure 1. Comparison of the replicate responses of (a) metal ion sensors, CO<sub>2</sub> and humidity sensors and (b) metal oxide sensors to five replicates of blank (B) medium and a treatment species after 72 hrs growth. The dotted lines indicate the *T. mentagrophytes* (M) treatment responses.

Figure 2. The temporal production of CO<sub>2</sub> for some treatments over the time scale of the experimental period.

Figure 3. The PCA map of the data for three *Trichophyton* species achieved using the electronic nose based on 14 conducting polymer sensor array system after 96 hrs incubation. There is little discrimination between the fungal species. Key to treatments: B1-B5, blank agar medium; M1-M5, *T. mentagrophytes*; R1-R5, *T. rubrum*, V1-V5, *T. verrucosum*.

Figure 4. The PCA map of the data of three *Trichophyton* species and the blank treatments after 96 hrs using the metal oxide/ion based electronic nose. This shows clear differentiation between the species examined on SABHI medium at 25°C. Incubation was for 1 hr in sample chambers before headspace analysis. Key to treatments: B, blank agar medium; M, *T. mentagrophytes*; R, *T. rubrum*, V, *T. verrucosum*.

Figure 5. The PCA map showing the discrimination between four species of *Trichophyton* species and the blanks after 96 hrs incubation on SDA at 25°C. Samples incubated for 1 hr in sample chambers prior to headspace analyses. Key to treatments: B, blank agar medium; M, *T. mentagrophytes*; R, *T. rubrum*, VE, *T. verrucosum*; VI, *T. violaceum*. This is based on using seven selected sensors).

Figure 6. Dendrogram showing the Cluster analysis and separation of the four *Trichophyton* species and the blanks after 96 hrs on SDA medium using Wards method and the Euclidian distances between samples. Key to treatments: B, Blank; VI, *T. violaceum*; VE, *T. verrucosum* and; R, *T. rubrum*; M, *T. mentagrophytes*.

Figure 7. PCA map of treatments of *T. mentagrophytes* of different initial concentrations (log1-log7) grown for 96hrs at 25°C on a solid agar medium.

Figure 8. Dendrogram showing the Cluster analysis and separation of the different initial concentrations (log1 to log7) of *T. mentagrophytes* after 72 hrs at 25°C using Wards method and the Euclidian distances between samples. Key to treatments: B, Blank; 1, log1; 3, log3, 5, log5, 7, log7.