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Arthroconidia production in *Trichophyton rubrum* and a new *ex vivo* model of onychomycosis

S. Amir Yazdanparast[1]† and Richard C. Barton[2]

[1]Department of Microbiology, University of Leeds, Leeds LS2 9JT, UK

[2]Mycology Reference Centre, Department of Microbiology, Leeds General Infirmary, Leeds LS1 3EX, UK

Correspondence
Richard C. Barton
Richard.Barton@leedsth.nhs.uk

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The dermatophyte fungus *Trichophyton rubrum* often produces arthroconidia *in vivo*, and these cells are thought to be involved in pathogenesis, and, in shed skin scales, to act as a source of infection. The purpose of this study was (i) to examine the environmental and iatrogenic factors which affect arthroconidiation in *T. rubrum in vitro*, (ii) to look at arthroconidia formation in a large number of clinical isolates of *T. rubrum* and (iii) to develop a new model for the study of arthroconidia formation in nail tissue. Arthroconidia production was studied in *T. rubrum* grown on a number of media and under conditions of varying pH, temperature and CO_2 concentration. The effect of the presence of antifungals and steroids on arthroconidia formation was also examined. Nail powder from the healthy toenails of volunteers was used as a substrate for arthroconidial production. On Sabouraud dextrose agar in the presence of 10 % CO_2 plus air, arthroconidial formation occurred optimally at 37 °C and pH 7·5, and was maximal at 10 days. Most isolates of *T. rubrum* showed a similar level of arthroconidial production, and only two out of 50 strains were unable to produce arthroconidia. Subinhibitory levels of some antifungals and betamethasone resulted in the stimulation of arthroconidia formation. Arthroconidial production in ground nail material also occurred under the same optimal conditions, but took longer to reach maximal levels (14 days). These *in vitro* and *ex vivo* results provide a useful basis for the understanding of arthroconidium formation *in vivo* in infected tissues such as nails.

INTRODUCTION

The high incidence of dermatophyte infections makes them an important public health problem (Aly, 1994), and studies estimate that between 10 and 15 % of the population worldwide is affected (Matsumoto, 1996). In spite of rapid improvements in treatment during the past decade, many aspects of dermatophyte infections remain poorly understood. Dermatophyte infection of the nail, known as tinea unguium or onychomycosis, is perhaps the most refractory to treatment, and requires several months of systemic therapy, with terbinafine being the most effective agent (Evans, 1999), even though a success rate of greater than 80–85 % cannot be expected. Possible reasons for the 20 % failure rate include the development of antifungal resistance, poor pharmacokinetics due to the characteristics of the nails (i.e. excessive thickness) and the presence of resistant structures such as subungual dermatophytomas (Evans & Roberts, 1998), and arthroconidia, which are frequently seen in infected nails, and whose role in disease has long been debated (Chin & Knight, 1957). Arthroconidium formation, characteristic of dermatophyte infection of skin, hair or nails, results from the fragmentation of a hypha following multiple septum formation. Growth in the stratum corneum of skin, nails and hair occurs in the form of hyphae, which may then form arthroconidia. It has been suggested that arthroconidia are more commonly found in wet skin lesions and nails (Miyazi *et al.*, 1971). The potential of arthroconidia to contribute to resistance to treatment is suggested by the fact that having no immediate exogenous nutritional requirements, they are resistant to adverse environmental conditions (Hashimoto & Blumenthal, 1978). Arthroconidia shed from lesions are likely to be the form involved in the spread of infection. The development of arthroconidia in *Trichophyton mentagrophytes* has been observed *in vitro* by a number of authors (Bibel *et al.*, 1979; Emyanitoff & Hashimoto, 1979; Weigl & Hejtmanek, 1979; Wright *et al.*, 1984) and in *Trichophyton rubrum* (Miyaji & Fujiwara, 1971). Here we describe a detailed examination of the environmental and iatrogenic factors which affect arthroconidiation *in vitro* in *T. rubrum*, and describe a new *ex vivo* model for the study of arthroconidium formation in nails.

METHODS

Organism. A total of 50 strains of *T. rubrum*, isolated from the skin

†Present address: Department of Mycology, Iran University of Medical Sciences, PO Box 15875-6171, Tehran, Iran.

and nails of patients, were used. Cultures were maintained at 27 °C by monthly subculture on Sabouraud dextrose agar (SDA), and permanent cultures were stored at −70 °C in 1·6 % DMSO.

Media and culture conditions. For arthroconidium formation, a standard pH of 5·6 was employed for SDA (Oxoid); other pHs were obtained by the addition of sodium hydroxide. When medium without glucose was required, peptone agar (Difco) at 1, 3, 5 and 10 % was used. Brain heart infusion (BHI, Oxoid), Trichophyton no. 1 (Difco), and SDA plus 1, 3, 5 and 10 % NaCl, were also used. Betamethasone 17-valerate, amphotericin B, itraconazole, griseofulvin and terbinafine were added to SDA to investigate their effects on arthroconidium formation. Each antifungal was dissolved in DMSO, and DMSO at the same concentration was used as a control. Cultures were incubated for up to 10 days at various temperatures, either under aerobic conditions or in air plus various CO_2 concentrations using a LEEC CO_2 incubator in which the CO_2 concentration was set and controlled automatically by the incubator.

Microscopic examination. Adhesive tape was cut in to 1 cm squares, and was placed with sterile forceps on the surface of a colony, lifted off, and mounted colony side up, and a drop of 70 % ethanol and a drop of lactophenol cotton blue were placed on top. The number of arthroconidia was determined after examining four randomly selected fields of view under ×400 magnification. Arthroconidia were defined as any hyphal compartment <4 μm long or any spherical cell representing a disarticulated arthroconidium that was not obviously an aleuroconidium (microconidium).

Ground nails for *ex vivo* experiment. Clippings from several healthy toenails previously found free from fungi when examined in 10 % KOH were cleaned with 70 % ethanol, dried in a sterile Petri dish at 37 °C, and ground with liquid nitrogen to a powder with a pestle and mortar. Plates of *T. rubrum* on SDA grown aerobically were harvested at 2 weeks by gently scraping the surface of the agar with a glass rod. The cells were suspended in distilled water, the pH was adjusted to 7·5 with NaOH, and the concentration of microconidia was standardized at 1×10^7 cells ml^{-1}. Microconidial suspension of *T. rubrum* (20 μl) was spread over the surface of 0·2 g nail powder on a sterile microscope slide, and the inoculated slide placed on a glass rod within a sterile glass Petri dish. Sterile water was added to the Petri dish to maintain a humid atmosphere, and then incubatation was carried out aerobically or in 10 % CO_2 at 37 °C for 14 days. The powder was then mounted, and examined microscopically in 10 % KOH, with or without lactophenol cotton blue staining.

RESULTS AND DISCUSSION

Factors affecting arthroconidium formation in *T. rubrum*

Arthroconidium formation occurred in all five isolates of *T. rubrum* used in preliminary studies, when 10 % of the incubation atmosphere was replaced by CO_2 on SDA media at 37 °C after 10 days. These conditions were used as standard in the comparison of the effect of different factors on arthroconidium formation. When cultured in normal air, the frequency of arthroconidium formation was zero. Analysis of the time-course of arthroconidium production in *T. rubrum* N250 showed that optimal arthroconidium formation was at 10 days (Fig. 1), and the optimal percentage of CO_2 for arthroconidium formation in *T. rubrum* N250 was 10 % (Fig. 2). Arthroconidium production in *T. mentagrophytes in vitro* at 31 °C is also induced by

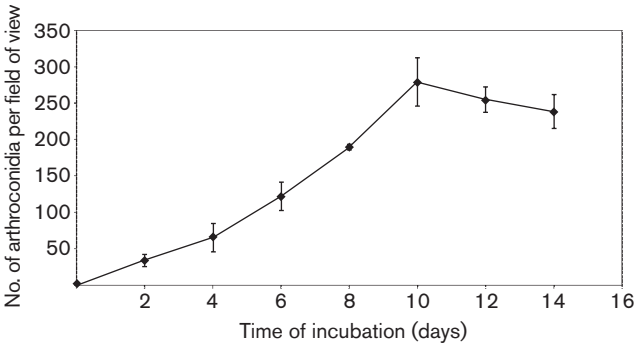

Fig. 1. Time-course of arthroconidia formation in *T. rubrum* N250 on SDA at 37 °C and 10 % CO_2. Means and 95 % confidence limits are shown ($n = 3$).

cultivation in an atmosphere with higher CO_2 tension (Bibel *et al.*, 1979). Curiously, Gupta *et al.* (2003) have found that *Trichophyton raubitschekii*, which some authors consider to be conspecific with *T. rubrum*, produces arthroconidia under normal aerobic conditions, and that this is not stimulated by 10 % CO_2. Whether the stimulated arthroconidiogenesis is due to the presence of CO_2 itself or reduced oxygen tension has been the subject of some controversy (Barrera, 1986). Some investigators (Emyanitoff & Hashimoto, 1979) have shown that exogenous CO_2 is not the absolute requirement, and that replacement of air by either N_2 or CO_2 stimulates arthrosporulation in *T. mentagrophytes* to the same extent. However, King *et al.* (1976) have shown that while helium and methane, like CO_2, tend to inhibit the growth of *T. mentagrophytes*, they do not induce arthroconidial formation. CO_2 diffuses physiologically from normal skin (Frame *et al.*, 1972), and its level rises in damaged or occluded skin (Malten & Thiele, 1973). Thus a rise in CO_2 tension together with a reduction in oxgen tension during infection may cause the transformation of dermatophyte hyphae to arthroconidium chains (Allen & King, 1978).

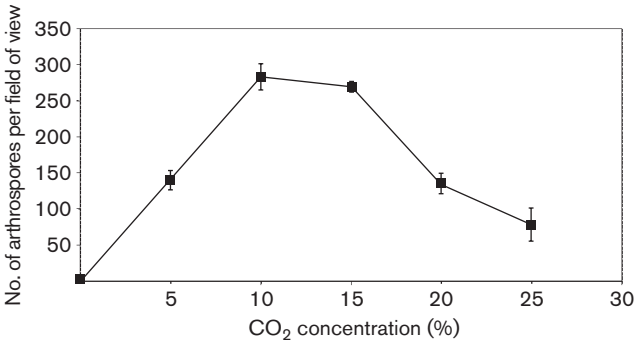

Fig. 2. Effect of CO_2 concentration on arthroconidia formation in *T. rubrum* N250 after 10 days on SDA at 37 °C. Means and 95 % confidence limits are shown ($n = 3$).

Table 1. Effect of five different media on arthroconidial production in five strains of *T. rubrum*

The mean and 95 % confidence limits are shown ($n=3$).

Strain	Medium				
	BHI agar	Trichophyton agar no. 1	SDA	SDA + 1 % NaCl	1 % peptone agar
N250	95·0 ± 17·4	20·0 ± 7·4	283·0 ± 17·4	185·0 ± 11·4	110·0 ± 10·8
N251	75·0 ± 19·7	45·0 ± 10·8	269·7 ± 5·2	160·0 ± 41·8	115·0 ± 11·4
15477	80·0 ± 13·8	59·0 ± 12·4	180·0 ± 4·3	130·0 ± 10·8	85·0 ± 23·7
14686	77·0 ± 24·4	34·0 ± 13·1	160·0 ± 11·4	155·0 ± 11·4	150·0 ± 227
13626	60·0 ± 5·0	25·0 ± 25·9	268·7 ± 10·0	140·0 ± 11·4	103 ± 16·3

In the presence of 10 % CO_2, SDA was the optimal medium for arthroconidium formation for most strains. No growth was seen in media containing > 3 % NaCl or > 3 % peptone, and the addition of 1 % NaCl did not increase arthroconidium formation (Table 1). Levels of arthroconidium formation were reduced even further with other media. Miyazi & Fujiwara (1971) have reported that *T. rubrum* produces a greater number of spherical cells (arthroconidia) on BHI at 37 °C than on other media, and that the presence of 0·85 % NaCl or ornithine is important for arthrosporulation.

When cultures were incubated at various temperatures with 10 % CO_2, a temperature of 37 °C was found to be maximal for arthroconidium formation (Fig. 3). At 42 °C, no growth occurred. Temperatures between 32 and 39 °C also strongly favour the arthroconidiation of *T. mentagrophytes* on SDA, and no or less arthroconidium formation is seen in *T. mentagrophytes* at 30 °C (Emyanitoff & Hashimoto, 1979); in addition, arthroconidium formation in *T. rubrum* was seen at 37 °C but not at 25 °C by Miyazi & Nishimura (1971). The temperature required for optimal arthroconidium formation *in vitro*, 37 °C, is higher than those recorded for the surface of the human body, typically 33 ± 1 °C, and near the upper limit for growth for this species. Although, generally, slightly acidic conditions favoured arthrosporulation, the optimal pH for arthroconidium formation was found to be 7·5 (Fig. 4). The optimal pH of the medium for arthroconidium formation in *T. rubrum* was found to be 7·4 by Miyazi *et al.* (1971).

Effect of antifungal agents and betamethasone on arthroconidia formation

Subinhibitory levels of several antifungal agents were tested for their effects on arthroconidium formation on SDA at 37 °C. At subinhibitory concentrations, some of these antifungal agents, including amphotericin B, griseofulvin and clotrimazole, stimulated the level of arthrosporulation, but itraconazole, and particularly terbinafine, suppressed arthroconidium production (Table 2). Similar effects have been seen in *T. mentagrophytes*, in which griseofulvin (0·5 μg ml^{-1}), clotrimazole (0·1 μg ml^{-1}), and particularly amphotericin B (5 μg ml^{-1}), stimulate a low level of arthrosporulation (Emyanitoff & Hashimoto, 1979). Compared to other antifungals, itraconazole, and particularly terbinafine, have significantly increased rates of cure and shorter treatment times (Roberts, 1999), and interestingly, in our study, only itraconazole, and particularly terbinafine, suppressed arthroconidium production in *T. rubrum* N250. These results may be clinically significant: if sufficiently fungicidal concentrations are not attained in some areas of the lesion, some antifungals may enhance the conversion of hyphae to arthroconidia. Hashimoto &

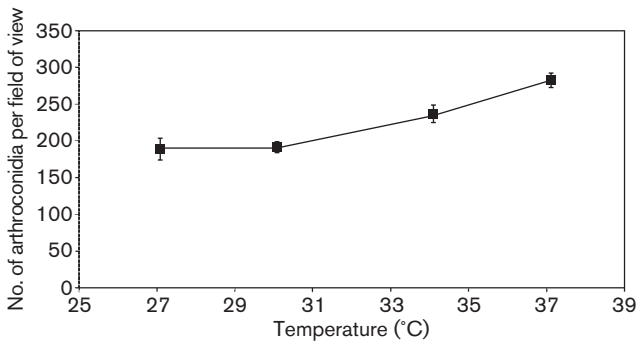

Fig. 3. Effect of temperature on arthroconidia formation in *T. rubrum* N250 after 10 days on SDA at 37 °C and 10 % CO_2. Means and 95 % confidence limits are shown ($n=3$).

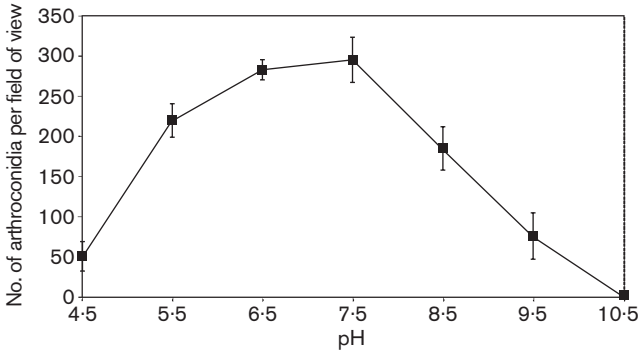

Fig. 4. Effect of pH on arthroconidia formation in *T. rubrum* N250 after 10 days on SDA at 37 °C and 10 % CO_2. Means and 95 % confidence limits are shown ($n=3$).

Table 2. Effect of different antifungals on *T. rubrum* N250

NA, Not applicable.

Antifungal	Concentration ($\mu g\ l^{-1}$)	Mean no. arthroconidia per field of view*
Amphotericin B	1·0	395 ± 5·4
	5·0	340 ± 9·3
Griseofulvin	0·25	364 ± 11·3
	0·5	295 ± 14·2
Clotrimazole	0·1	373 ± 6·1
	0·5	314 ± 8·8
Itraconazole	0·25	210 ± 10·1
	0·5	175 ± 17·7
Terbinafine	0·001	120 ± 8·1
	0·05	95 ± 7·3
DMSO (10 %)	NA	275 ± 19·3
Sabouraud broth control	NA	280 ± 5·4

*The mean and 95 % confidence limits are shown ($n = 3$).

Blumenthal (1978) have reported that dormant arthroconidia of *T. mentagrophytes* are resistant to common antimycotics such as griseofulvin, clotrimazole, miconazole and nystatin. This resistance may be due to the nature of the arthroconidial wall. The steroid betamethasone 17-valerate at 1 mg ml^{-1} caused appreciable stimulation of arthroconidium formation (352 ± 2·6 arthoconidia per field of view) compared to the control (255 ± 2·2) (mean of three experiments ± SD). An early report (Chattaway *et al.*, 1959) showed an inhibition of the growth of *T. rubrum* by different steroids. Thus, steroids may influence either directly or indirectly the development of dermatophyte infections.

Clinical isolates of *T. rubrum*

Of a total of 50 clinical isolates, obtained before, during and after treatment with itraconazole and terbinafine, only two strains were unable to produce arthroconidia under the previously defined optimal conditions. Of these isolates, 45 were from nine patients (two to six per patient), and therefore not necessarily independent isolates. However, the two isolates that failed to produce arthroconidia came from patients from whom arthroconidium-producing isolates had also been obtained. This might represent the suppression of the ability to produce arthroconidia in a strain during treatment or might represent different strains, since most toenail infections caused by *T. rubrum* have recently been shown to be caused by more than one strain (Yazdanparast *et al.*, 2003). Fujita *et al.* (1986) found that two of eight strains of *T. mentagrophytes* did not produce arthroconidia; also, eight of 15 strains of *T. rubrum* examined by Miyazi & Nishimura (1971) failed to produce arthroconidia. This discrepancy may relate to differences in methodology.

Arthroconidial formation in *ex vivo* nails

Arthroconidial production in ground nails from the toenails of volunteers occurred under the same optimal conditions, and again did not occur unless the atmosphere was replaced with 10 % CO_2, although it took longer (14 days) to reach maximal levels. This suggests that during invasion of the nail by hyphal growth, changes to the environment of the fungus caused by the reaction of the host tissue to the fungus induce the formation of arthroconidia. For example, hyperkeratosis, often seen in onychomycosis, may lead to a decrease in local O_2 concentration and/or increase in CO_2 concentration, as seen in damaged skin (Malten & Thiele, 1973). It has recently been demonstrated in a similar nail model (though without arthroconidial formation) that the mimimum fungicidal concentration of terbinafine against *T. rubrum* within nails is increased up to 100-fold compared with standard media (Osborne *et al.*, 2004). This suggests, in general, that the properties of this dermatophyte in nail models are likely to differ significantly from those of fungi grown on laboratory media and to be more representative of the situation *in vivo*.

It has yet to be demonstrated that arthroconidia of *T. rubrum* formed *in vitro*, and therefore presumably *in vivo*, are more resistant to antifungal activity than hyphae. Terbinafine, as one of the main therapeutic agents for onychomycosis, has been demonstrated to be fungicidal, though this has only been shown using actively growing hyphae (Hazen, 1998). The thick walls of arthroconidia may resist antifungal treatment, only to germinate following the cessation of treatment. Indeed, an approach designed to stimulate the germination of resistant propagules in onychomycosis patients who failed antifungal therapy by applying slides of Sabouraud agar to diseased nails for 24 h prior to topical antifungal treatment has been shown to have a high cure rate (Pierard *et al.*, 2000).

In conclusion, this study has shown how certain conditions, including physiological changes within the nail and antifungal agents, may trigger the formation of arthroconidia from hyphal cells, and that a key factor is the presence of 10 % CO_2. Further work is needed to examine the widely assumed importance of the role of arthroconidia in pathogenesis and the resistance of dermatophytes to effective antifungal treatment.

REFERENCES

Allen, A. M. & King, R. D. (1978). Occlusion, carbon dioxide and fungal skin infections. *Lancet* **1**, 360–362.

Aly, R. (1994). Ecology and epidemiology of dermatophyte infections. *J Am Acad Dermatol* **31**, S21–S25.

Barrera, C. R. (1986). Formation and germination of fungal arthroconidia. *CRC Crit Rev Microbiol* **12**, 271–292.

Bibel, D. J., Crumrine, D. A., Yee, K. & King, R. D. (1979). Development of arthrospores of *Trichophyton mentagrophytes*. *Infect Immun* **15**, 958–971.

Chattaway, F. W., Townsley, J. D. & Barlow, A. J. E. (1959). Effect of steroids and related compounds on the growth of dermatophytes. *Nature* **184**, 1731.

Chin, B. & Knight, S. G. (1957). Growth of *Trichophyton mentagrophytes* and *Trichophyton rubrum* in increased carbon dioxide tensions. *J Gen Microbiol* **16**, 642–646.

Emyanitoff, R. G. & Hashimoto, T. (1979). The effects of temperature, incubation atmosphere, and medium composition on arthrospore formation in the fungus *Trichophyton mentagrophytess*. *Can J Microbiol* **25**, 362–366.

Evans, E. G. V. (1999). Causative pathogens in onychomycosis and the possibility of treatment resistance: a review. *J Am Acad Dermatol* **38**, S32–S36.

Evans, E. G. V. & Roberts, D. T. (1998). Subungual dermatophytoma complicating dermatophyte onychomycosis. British Association of Dermatologists. *Br J Dermatol* **138**, 189–203.

Frame, G. W., Straus, W. G. & Mailbach, H. I. (1972). Carbon dioxide emission of the human arm and hand. *J Invest Dermatol* **59**, 155.

Fujita, S., Matsuyama, T. & Sato, Y. (1986). A simple and reliable culturing method for production of arthroconidia by dermatophytes. *Jpn J Med Mycol* **27**, 175–181.

Gupta, A. K., Ahmad, I., Porretta, M. & Summerbell, R. C. (2003). Arthroconidial formation in *Trichophyton raubitschekii*. *Mycoses* **46**, 322–328.

Hashimoto, T. & Blumenthal, H. J. (1978). Survival and resistance of *Trichophyton mentagrophytes* arthrospores. *Appl Environ Microbiol* **35**, 274–277.

Hazen, K. C. (1998). Fungicidal versus fungistatic activity of terbinafine and itraconazole: an in vitro comparison. *J Am Acad Dermatol* **38**, S37–S41.

King, R. D., Dillavou, C. L., Greenberg, J. H., Jeppsen, J. C. & Jaeger, J. S. (1976). Identification of carbon dioxide as a dermatophyte inhibitory factor produced by *Candida albicans*. *Can J Microbiol* **22**, 1720.

Malten, K. E. & Thiele, F. A. J. (1973). Evaluation of skin damage. II – water loss and carbon dioxide release measurements related to skin resistance measurements. *Br J Dermatol* **89**, 565–569.

Matsumoto, T. (1996). Fungal diseases in dermatology. In *Principles and Practice of Clinical Mycology*, pp. 105–129. Edited by C. C. Kibbler, D. W. R. Mackenzie & F. C. Odds. Chichester: Wiley.

Miyaji, M. & Fujiwara, K. (1971). Effect of amino acids, salt, and pH on the spherical cell formation. *Jpn J Med Mycol* **12**, 200–205.

Miyazi, M. & Nishimura, K. (1971). Studies on arthrospore of *T. rubrum*. *Jpn J Med Mycol* **12**, 18–23.

Miyazi, M., Nishimura, K. & Kariya, H. (1971). Relationship between the types of eruption and the parasitic form of *T. rubrum*. *Jpn J Med Mycol* **12**, 81–85.

Osborne, C. S., Leitner, I., Favre, B. & Ryder, N. S. (2004). Antifungal drug response in an *in vitro* model of dermatophyte nail infection. *Med Mycol* **42**, 159–163.

Pierard, G. E., Pierard-Franchimont, C. & Arrese, J. E. (2000). The boosted antifungal topical treatment (BATT) for onychomycosis. *Med Mycol* **38**, 319–392.

Roberts, D. T. (1999). Onychomycosis: current treatment and future challenges. *Br J Dermatol* **141** (Suppl. 56), 1–4.

Weigl, E. & Hejtmanek, M. (1979). Differentiation of *Trichophyton mentagrophyte*s arthrospores controlled by physical factors. *Mykosen* **22**, 167–172.

Wright, L. R., Scott, E. M. & Gorman, S. P. (1984). Spore differentiation in a clinical strain of *Trichophyton mentagrophytes*. *Microbios* **39**, 87–93.

Yazdanparast, A., Jackson, C. J., Barton, R. C. & Evans, E. G. V. (2003). Molecular strain typing of *Trichophyton rubrum* indicates multiple strain involvement in onychomycosis. *Br J Dermatol* **148**, 51–54.