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The hair strand test—A new method for testing antifungal effects of antidandruff preparations

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Synopsis

Seborrheic dermatitis and its minimal variant, dandruff (pityriasis simplex capillitii), are among the most frequent diseases caused by *Malassezia* (*M.*) yeasts. Treatment studies have shown that antimycotics achieve clinical improvement, while recolonization leads to recurrent symptoms. Among the antimycotics used are azoles, hydroxypyridones, and various agents such as zinc pyrithione, tar, and selenium disulfide. However, comparative efficacy studies *in vitro* should not only consider the minimal inhibitory concentrations against *Malassezia* yeasts but also the bioavailability of the individual substances with regard to hair and scalp. By means of a new method, the hair strand test, hairs from ten volunteers were subjected to standardized 5-min incubation with different shampoo formulations. Thereafter they were rinsed with running water for 1 min and dried. Two hundred each of these hairs (length 1 cm) were given into a medium (olive oil on selective agar for pathogenic fungi) inoculated with *M. sympodialis* or *M. globosa* (5×10^3 CFU/µl), and the influence on growth was semiquantitatively determined over a period of up to 18 days. According to preliminary results, 1% climbazole proved to be particularly effective. The hair strand test, which can also be performed *ex vivo,* is a new method to find out whether antimycotic agents bind differently to the hair substance and, via a depot effect, may influence the growth of *Malassezia* yeasts and thus affect dandruff. This allows conclusions about the efficacy of antidandruff formulations.

INTRODUCTION

Yeasts of the genus *Malassezia* (*M.*) are part of the human microflora and that of several homeotherms (1,2). As their growth depends on an exogenous lipid source, they are primarily found in seborrheic areas that are rich in sebaceous glands. Seven species are currently differentiated: *M. furfur, M. pachydermatis, M. sympodialis, M. globosa, M. obtusa, M. restricta* and *M. slooffiae* (3,4). Initial epidemiological studies have shown that *M. sympodialis* and *M. globosa* are among the most frequent species on human skin (1–4).

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Apart from their significance as saprophytes, *Malassezia* spp. can cause diseases. In addition to pityriasis versicolor, seborrheic dermatitis and its minimal variant, dandruff (pityriasis simplex capillitii), are the most common (1,5). Although some environmental factors should not be overlooked, *Malassezia* spp. are the main protagonists of dandruff as a reactive response of the scalp epidermis (6).

The relationship between *Malassezia* and dandruff was demonstrated *ex juvantibus* in a series of treatment studies that showed that antimycotics achieved clinical improvement while recolonization resulted in reoccurrence of symptoms (5–10). However, it is still unknown in which way these yeasts cause skin diseases (5). Increased growth cannot be the only reason. Some studies denied a constantly and significantly increased yeast carriage in patients compared with healthy individuals (14–16), while others did not (8,11–13). Therefore, qualitative rather than quantitative changes in the resident scalp flora (*Malassezia* spp., aerobic cocci, corynebacteria) appear to be significant. McGinley *et al.* (17) found a 100% incidence of *Malassezia* in dandruff patients compared to 98% in individuals with healthy skin. However, there was a decisive difference in the portion of *Pityrosporum/Malassezia* on the scalp flora: 46% in healthy persons vs 74% in dandruff patients and 82% in those with seborrheic dermatitis. The geometric mean value for microorganisms per cm^2 was 5.05×10^5 in healthy individuals, 9.22×10^5 in dandruff patients, and 6.45×10^5 in those with seborrheic dermatitis ($p < 0.05$).

Among the antimycotics, azoles (climbazole, clotrimazole, ketoconazole, oxiconazole), polyenes (amphotericin B), hydroxypyridones (ciclopirox, octopirox), and various other preparations, such as zinc pyrithione, coal tar, selenium disulfide and polidocanol, are used (5,6,10,18). Comparative studies *in vitro* should not only consider the minimal inhibitory concentrations of the individual agents against *Malassezia* yeasts, but also their bioavailability in the target compartment (hair and scalp). By means of a simple method using the presented hair strand test it was investigated whether antimycotic agents bind differently to the hair substance and via a depot effect influence the growth of *Malassezia* yeasts. This would allow further conclusions about the efficacy of antidandruff preparations.

EXPERIMENTAL METHODS

MALASSEZIA SPECIES

As it is still unknown which *Malassezia* spp. are involved in the development of dandruff, reference strains of the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands, were used for *M. sympodialis* (CBS 7222) and *M. globosa* (CBS 7966). These two species are most commonly found on the scalp (2).

Hair specimens were taken from ten volunteers of different hair color (six female, four male; mean 28.2 years, 5–53 years), who did not use antidandruff preparations or hair dyes. By means of scissors the strands were cut near the scalp surface (hair roots were not included in the sample). Five different shampoo formulations were used, blinded by the manufacturer (Beiersdorf AG, Hamburg, FRG): (A) anti-dandruff shampoo (2% polidocanol + 0.5% octopirox + 1% climbazole); (B) shampoo base + 2% polidocanol; (C) shampoo base + 0.5% octopirox; (D) shampoo base + 1% climbazole; and (E) shampoo base (INCI: aqua, sodium laureth sulfate, undecylenamidopropyl betaine, laureth-9,

sodium chloride, trideceth-2 carboxamide MEA, citric acid, sodium benzoate, PEG-200 hydrogenated glyceryl palmate, sodium salicylate, and polyquaternium-10).

STRUCTURE OF THE TRIAL

Sterile glass Petri dishes (3 cm in diameter) were filled with 4 ml of selective agar for pathogenic fungi (SPF; Merck, Darmstadt, FRG). Cold-sterilized olive oil was inoculated with the different *Malassezia* strains, which were cultured for four days on SPF overlaid earlier with olive oil, and adjusted to an inoculation density of 5×10^3 CFU/µl according to McGinley *et al.* (17) using a Neubauer chamber (18). Two-hundred microliters of this suspension were pipetted into the prepared Petri dishes so as to cover an area of about one square centimeter (~10^6 CFU/cm^2).

A model to imitate hair washing procedure was developed as follows: From each volunteer, hair strands approximately 5 cm in length were incubated with one of the five test substances at 30°C for 5 min in sterile Petri dishes. The hairs were then transferred to a sieve with filter paper, rinsed for 1 min in running water (30°C), and dried at room temperature. By means of sterile scissors, 1-cm pieces were cut from the dried hair and distributed in the center of the different test dishes. To approximate natural scalp conditions, 200 hairs/cm^2 were inoculated. Growth of *Malassezia* yeasts as compared with a positive control (200-µl inoculation suspension without addition of hair) was evaluated as follows: + = growth, (+) = weak growth, and 0 = no growth after incubation at 30°C. Two hundred microliters of pure olive oil on SPF with addition of hair was used as a negative control. The trials were performed two times.

STATISTICAL ANALYSIS

The trial was a single-blind, vehicle-controlled *in vitro* study. The individual test formulations were compared in pairs by means of the McNemar test at a local level of 5%.

RESULTS

Different results were obtained with regard to test formulations, test hairs, and test strains. *M. sympodialis* showed faster growth in the control, so that a first evaluation was already possible after four days. As growth of *M. globosa* is known to be significantly slower (3), the dishes could not be evaluated until the fifteenth day. The results are shown in Table I. Repeated testing revealed no differences. Hair strands incubated in pure olive oil on SPF showed no growth at any time.

Individual evaluations for *M. sympodialis* after four-day incubation are shown in Table II. They were nearly identical for test substances B, C, and E. All ten hair specimens that had been treated with these preparations showed growth of *M. sympodialis* after four days. In some of them, growth was only observed in the marginal region, i.e., in an area where there was no direct contact with the inoculated hairs. With increasing incubation time, however, homogenous growth was observed. The results of paired comparisons of test formulations A/D and B/C/E are summarized in Table III. The difference between formulations A and D was found to be insignificant. All other paired comparisons of formulations A and D showed statistically relevant differences—after four days, prepa-

Table I
Growth of *M. sympodialis* and *M. globosa* in Different Test Preparations After Varying Incubation Times

		Frequency of growth (of n = 10) with			
		M. sympodialis/incubation time (days)		*M. globosa*/incubation time (days)	
Code	Substance	4	11	15	18
A	Antidandruff shampoo	3	7	1	1
B	Shampoo base + 2% polidocanol	10	10	7	7
C	Shampoo base + 0.5% octopirox	10	10	8	9
D	Shampoo base + 1% climbazole	4	5	1	1
E	Shampoo base	10	10	8	8

Because of slower growth, *M. globosa* could not be evaluated prior to day 15.

Table II
Results of Hair Strand Test for *M. sympodialis* After Four Days

Hair from volunteer	Shampoo A	Shampoo B	Shampoo C	Shampoo D	Shampoo E
1	+ (margin)	+	+	+	+
2	0	+	+	0	+ (margin)
3	0	+	+	0	+
4	0	+	+	0	+
5	+	+	+	0	+
6	0	+ (margin)	+ (margin)	0	+ (margin)
7	0	+	+	0	+
8	0	+	+	+	+
9	+	+	+	+	+
10	0	+	+	+	+
Growth/total number	3/10	10/10	10/10	4/10	10/10

Margin = growth only at the margin of the suspension inoculate where there is no hair.

rations A and D were significantly better than B, C, and E. It was not possible to calculate *p*-values for paired comparisons of test formulations with identical results in the hair strand test. For these comparisons, the McNemar test results are shown in Tables IV and V. It should also be noted that the same *p*-values occur for different comparisons (e.g., 0.016 or 0.031). This phenomenon results from the small number of cases and the discrete character of distribution. After 11-day incubation, the test results remained unchanged for preparations B, C, and E. The number of specimens showing growth increased to seven with A and five with D (see Table I). Therefore, the McNemar test failed to reveal statistically significant differences among the five test substances after eleven days of incubation.

Results of the hair strand test for *M. globosa* after 18 days are shown in Table III and Figures 1 and 2. As observed with *M. sympodialis,* preparations A and D had a significant growth-inhibiting effect. Only in one case each was growth of *M. globosa* observed. The McNemar test results are shown in Table V. No significant differences were found among preparations B, C, and E. Test formulations A and D were significantly better than preparations B, C, and E. After 18 days, the hair strand test revealed the same result for test preparations A, B, D, and E. The number of specimens showing growth with

Table III
Results of Hair Strand Test for *M. globosa* After 18 Days

Hair from volunteer	Shampoo A	Shampoo B	Shampoo C	Shampoo D	Shampoo E
1	0	+	+	0	+
2	0	+	+	0	+
3	0	(+)	+	0	+
4	0	+	0	0	+
5	+	+	+	0	+
6	0	0	+ (margin)	0	0
7	0	+	+	0	+
8	0	+	+	0	+
9	0	0	+	+	+
10	0	0	+	0	0
Growth/total number	1/10	7/10	9/10	1/10	8/10

Table IV
Results of McNemar Test for *M. sympodialis* After Four Days (p-Values)

Formulation	B	C	D	E
A	0.016	0.016	1.000	0.016
B	—	—	0.031	—
C	—	—	0.031	—
D	—	—	—	0.031

Table V
Results of McNemar Test for *M. globosa* After 18 Days (p-Values)

Formulation	B	C	D	E
A	0.031	0.008	1.000	0.016
B	—	0.625	0.070	1.000
C	—	—	0.008	1.000
D	—	—	—	0.016

preparation C increased from eight to nine (see Table I). The McNemar test results after 18 days were identical to those obtained after 15 days.

DISCUSSION

By means of the hair strand test, a new *in vitro* test system adapted to natural conditions in terms of inoculate, hair density, and application, it was shown that hair shampoos/substances have different antifungal effectiveness against *Malassezia* yeasts, which are thought to be primarily involved in the pathogenesis of dandruff (pityriasis simplex capillitii) (5,6,10). The test was unusual in that antifungal activity was not determined directly, but indirectly after incubation of hair samples in various test preparations to assess the bioavailability of the antifungal agents and a possible depot effect. Olive oil overlaid on SPF was used instead of Dixon's agar or Leemings's medium to provide a lipophilic environment resembling the sebum-rich milieu near the scalp surface. The

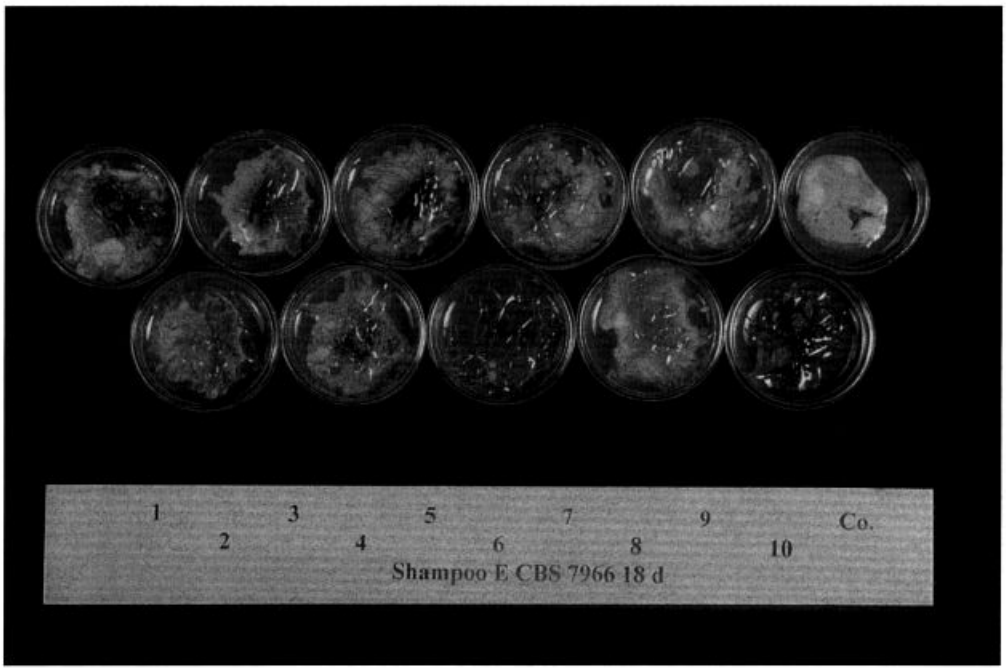

Figure 1. Growth of *M. globosa* CBS 7966 after 18-day incubation; hair from ten volunteers pre-incubated in shampoo base (E) (growth 8/10; Co = control).

miniaturized test system developed from preliminary studies was easy to perform and clear. The rate of contamination was low (1 of 240 samples contaminated with molds), and no growth of *Malassezia* was seen with the hair strands incubated in pure olive oil. In a few cases, evaluation was problematic because of "marginal" growth, i.e., slow growth in a part of the Petri dish that was not in direct contact with the incubated hairs. However, with increasing incubation time, complete growth occurred. In a blinded manner, shampoos were tested for growth of *M. globosa* and *M. sympodialis,* species that are frequently observed on the scalp. The antidandruff shampoo (preparation A; combination of all active agents) and the shampoo base with 1% climbazole (preparation D) were the most effective and significantly superior to shampoo base alone, while the latter was not different from the other test formulations. The strain of *M. globosa*, which showed slower growth than the strain of *M. sympodialis* (3), was somewhat more sensitive. In the case of *M. globosa,* the shampoo base was also effective to a certain extent, but it was not possible to distinguish between the base and the addition of polidocanol and octopirox.

Climbazole-containing preparations appear to have a predominantly fungistatic effect on *M. sympodialis,* which levels off with the longer incubation time of 15 days (particularly observed with antidandruff shampoo). However, as the user will probably have washed his/her hair in the meantime, this might be neglectable in practice. Such an effect is not demonstrable with *M. globosa.*

Synergistic effects of climbazole and other components of the antidandruff shampoo (polidocanol, octopirox) were not demonstrated in the present trial. The lacking effectiveness of octopirox, also as a single substance, in this test model may result from the

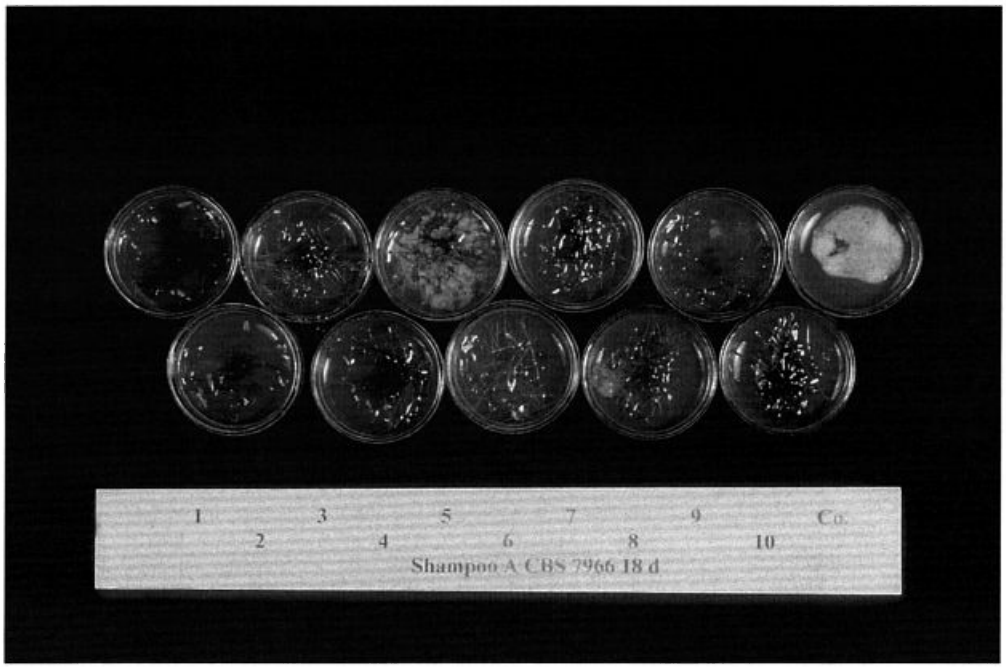

Figure 2. Growth of *M. globosa* CBS 7966 after 18-day incubation; hair from ten volunteers pre-incubated in andidandruff shampoo (A) (growth 1/10; Co = control).

markedly lower concentration compared with that of climbazole, but may also be a specific feature of the substance, which is revealed by the hair strand test. Previous *in vitro* studies have shown hydroxypyridones to be highly effective against *Malassezia spp.*, but data on their bioavailability in/on human hair are not yet available; this also applies to the other agents tested. The polidocanol concentrations used should actually have had an inhibitory effect (18), but probably this substance, too, does not bind to human hair, so that such an effect could not be demonstrated. Tests using higher concentrations might be reasonable.

CONCLUSION

In summary, the *in vitro* hair strand test was found to be an interesting and reliable new test model for evaluation of the antifungal activity of antidandruff preparations, especially with regard to a possible depot effect. Climbazole proved to be effective. With all other agents, no bioavailability from the hair was found, possibly because of the low concentrations used. Other substances (e.g., zinc pyrithione, ketoconazole, selenium disulfide, tar) are currently tested with the new system. The current test model does not primarily assess binding of antimycotics to scalp keratin. Active ingredients might rediffuse from the compartment of the hair to influence the growth of *Malassezia* yeasts on the sebum-rich scalp surface. Supplementary examination of the hair samples by GC-MS analysis would substantiate the validity of the test system. The hair strand test could also be performed *ex vivo* with hair samples from volunteers who regularly use

antidandruff preparations. In addition, the effects of repeated washing with or without active ingredients (wash-out kinetics, saturation effects) could also be evaluated.

REFERENCES

(1) E. Guého, T. Boekhout, H. R. Ashbee, *et al.,* The role of *Malassezia* species in the ecology of human skin and as pathogens, *Med. Mycol.,* **36** (suppl. 1), 220–229 (1998).
(2) G. Midgley, The lipophilic yeasts: State of the art and prospects, *Med. Mycol.,* **38** (suppl. 1), 9–16 (2000).
(3) E. Guého, G. Midgley, and J. Guillot, The genus *Malassezia* with description of four new species, *Antonie van Leeuwenhoek,* **69,** 337–355 (1996).
(4) J. Guillot, E. Guého, M. Lesourd, *et al.*, Identification of *Malassezia* species: A practical approach. *J. Mycol. Méd.,* **6,** 103–110 (1996).
(5) R. J. Hay and C. Graham-Brown, Dandruff and seborrheic dermatitis: Causes and management, *Clin. Exp. Derm.,* **22,** 3–6 (1997).
(6) C. Piérard-Franchimont, J. F. Hermanns, H. Degreef, and G. E. Piérard, From axioms to new insights into dandruff, *Dermatology,* **200,** 93–98 (2000).
(7) R. D. Aron-Brunetière, D. Dompmartin-Pernot, and E. Drouhet, Treatment of pityriasis capitis (dandruff) with econazole nitrate, *Acta Derm. Venereol.,* **57,** 77–80 (1977).
(8) L. C. Barber, The aetiology of dandruff. PR No. 8. *Pityrosporum ovale*/amphotericin B dandruff aetiology study (DA-125). Internal Report. Procter & Gamble, Cincinnati, 1977.
(9) M. M. Carr, D. Pryce, and F. A. Ive, Treatment of seborrheic dermatitis of the scalp with 2% ketoconazole, *Br. J. Dermatol.,* **116,** 213–216 (1986).
(10) S. Shuster, The aetiology of dandruff and mode of action of therapeutic agents, *Br. J. Dermatol.,* **111,** 235–242 (1984).
(11) M. C. Y. Heng, C. L. Henderson, D. C. Barker, *et al.,* Correlation of *Pityrosporum ovale* density with clinical severity of seborrhoeic dermatitis assessed by a simplified technique, *J. Am. Acad. Dermatol.,* **23,** 82–86 (1990).
(12) R. W. Van der Wyck and F. C. Roia, The relationship between dandruff and the microbial flora of the human scalp, *J. Soc. Cosmet. Chem.,* **15,** 761–768 (1964).
(13) R. W. Van der Wyk and K. E. Hechemy, A comparison of the bacterial and yeast flora of the human scalp and their effect upon dandruff production, *J. Soc. Cosmet. Chem.,* **18,** 629–639 (1967).
(14) I. M. Bergbrant and J. Faergemann, Seborrhoeic dermatitis and *Pityrosporum ovale*: A cultural and immunological study, *Acta Derm. Venereol.,* **69,** 332–335 (1989).
(15) D. C. Clift, H. J. Dodd, J. D. T. Kirby, *et al.,* Seborrhoeic dermatitis and malignancy. An investigation of the skin flora, *Acta Derm. Venereol.,* **68,** 48–52 (1988).
(16) N. J. Van Abbe, The investigation of dandruff, *J. Soc. Cosmet. Chem.,* **15,** 609–630 (1964).
(17) K. J. McGinley, L. J. Leyden, R. R. Marples, *et al.,* Quantitative microbiology of the scalp in non-dandruff, dandruff, and seborrheic dermatitis, *J. Invest. Dermatol.,* **64,** 401–405 (1975).
(18) P. Mayser and K. Gründer, Growth inhibition of *Malassezia* species by pharmacological concentrations of polidocanol, *Mycoses,* **38,** 23–27 (1995).