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The carriage of *Malassezia furfur* serovars A, B and C in patients with pityriasis versicolor, seborrhoeic dermatitis and controls

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Summary The aetiological role of *Malassezia furfur* in various dermatoses is controversial. The role of the three serovars of *M. furfur* in *Malassezia*-associated diseases has not been investigated. This study measured population densities of *M. furfur* serovars A, B and C, propionibacteria and *Micrococcaceae* on the chest, back, forehead, left and right cheeks of 10 patients with pityriasis versicolor, and 10 age- and sex-matched controls; and 10 patients with seborrhoeic dermatitis, and 10 age- and sex-matched controls. The population densities of *M. furfur*, propionibacteria and *Micrococcaceae* did not vary at a given site between patients and the corresponding control subjects. *Malassezia furfur* serovar A was found to be the predominant isolate on the chest and back of all four groups, but there was no difference in the distribution of serovars on the forehead and cheeks. No serovar was specifically associated with lesional skin in either disease. Thus, this data indicated that there was no difference in either the total population density of *M. furfur* or the distribution of serovars on lesional skin compared with control skin in either pityriasis versicolor or seborrhoeic dermatitis.

Malassezia furfur is a lipophilic, dimorphic yeast which is a saprophyte on human skin.[1] It occurs in highest density on areas which are rich in sebaceous glands, i.e. the chest, back and face.[2] The population density of *M. furfur* on any given individual varies with age.[3] Children as young as 1–2 months have been shown to be colonized.[4] The peak population density occurs between puberty and middle age, after which the population density declines.[5]

Previously, the designation *Malassezia* was used to describe the mycelial phase of the organism, whereas the yeast phase was divided into two distinct species on the basis of microscopic morphology:[6] *Pityrosporum orbiculare*, with round yeast cells and budding from a narrow neck; *Pityrosporum ovale*, with oval cells and budding from a wide neck. *Pityrosporum orbiculare* was widely regarded as the aetiological agent of pityriasis versicolor, but both variants are now designated *Malassezia furfur*.

The primary isolation of *M. furfur* was hampered for many years because of its fastidious nature and the lack of suitable recovery media. In 1987, Leeming and Notman developed a medium specifically for *M. furfur*, which they showed to be superior to other routinely used media.[7] They noted that the medium recovered several different colonial variants. Previous authors had also documented distinct variants which differed in physiology and antigenic composition.[8,9] In 1990, using the medium formulated by Leeming and Notman,[7] Cunningham *et al.*[10] observed and differentiated three distinct forms of *M. furfur*. They were different morphologically and physiologically, and serological studies demonstrated that they possessed distinct cell-surface antigens. The forms, or serovars, were designated A, B and C. The stability of the serovars, and the relationship to other classifications of *M. furfur* were discussed by Cunningham *et al.*[10]

Malassezia furfur has been associated with a variety of cutaneous and systemic diseases, as well as occurring as a skin commensal. These diseases include pityriasis versicolor, seborrhoeic dermatitis, folliculitis, peritonitis and catheter-related systemic infection. The aetiological role of *M. furfur* in some of these diseases is controversial. In seborrhoeic dermatitis, for example, evidence for the involvement of *M. furfur* comes from the favourable clinical response of the condition to anti-*Malassezia* agents.

The aim of this work was to study the cutaneous flora of patients with pityriasis versicolor, and patients with seborrhoeic dermatitis, in order to establish whether there was any association between the newly defined serovars of *M. furfur* and either of the diseases. In order to

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gain an overall picture of the flora, the study also examined the population densities of *Micrococcaceae* and propionibacteria, to determine whether there was any change in either of the diseases.

Methods

Reagents

Bacteriological peptone, yeast extract, ox bile, agar No. 1, blood agar base, Reinforced Clostridial Agar, and phosphate-buffered saline (PBS), were obtained from Oxoid Ltd. Glucose, glycerol, Tween 60, chloramphenicol, cycloheximide and furoxone were obtained from Sigma Chemical Co. Ltd, and glycerol monostearate from British Drug Houses. Serovaring of yeast strains was carried out on multispot microscope slides from C.A. Hendley Ltd, using fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit immunoglobulins from Dako Ltd.

Patients and controls

The patients studied were all attending the dermatology out-patient clinic at Leeds General Infirmary. There were 10 patients with pityriasis versicolor (mean age 34·0 years; range 23–41), and 10 patients with seborrhoeic dermatitis (mean age 34·9; range 25–46). None of the patients was receiving medication for their skin condition at the time of sampling, or had received treatment for at least 2 weeks previously. Age- and sex-matched controls had no previous history of either pityriasis versicolor or seborrhoeic dermatitis, and were not taking antimicrobial therapy at the time of sampling. The control group for the pityriasis versicolor patients had a mean age of 33·9 years (range 23–40), and the control group for the seborrhoeic dermatitis patients had a mean age of 34·3 years (range 26–45).

Estimation of microbial population densities

Microbiological sampling was carried out on each patient and each control at five standard sites, in triplicate. The sites sampled were the chest, back, forehead, left and right cheeks. These sites were sampled whether they were involved by the disease or not, but a record was kept of which sites were lesional. If sites were classed as lesional, the sampling ring was placed in such a way that the entire area sampled was lesional. Due to the extent of the disease on the patients sampled, if the site was lesional then all three replicate samples taken from that site were lesional. Sampling was carried out using the Williamson and Kligman technique,[11] with a ring of 1·767 cm^2 area, except in two patients, who were sampled using a ring of 4·909 cm^2 area. The controls for these patients were sampled using a ring of the same size. Ten-fold dilutions of the samples were made in half-strength wash fluid (PBS with 0·05% [v/v] Triton X-100, pH 7·9). The samples were cultured for *Micrococcaceae*, propionibacteria and *Malassezia furfur*. For the isolation of *Micrococcaceae*, three 20-μl drops of the appropriate dilutions were dried on to the surface of heated blood agar, and incubated at 37°C, aerobically, for 2 days. Propionibacteria were isolated by drying three 20-μl drops of appropriate dilutions on to Reinforced Clostridial Agar with furoxone,[12] and incubating in an anaerobic cabinet with a 10:10:80 CO_2:H_2:N_2 atmosphere at 37°C for 7 days. *Malassezia* were recovered by plating 100 μl of the appropriate dilutions on to the medium of Leeming and Notman,[7] and incubating at 34°C for 14 days, in a moist environment. Triplicate samples were taken at each site, and the number of colony-forming units/cm^2 skin (CFU/cm^2 skin) was calculated by taking the mean result of the three samples. For *Micrococcaceae* and the propionibacteria, the lower limit of detection of the sampling technique was 57 CFU/cm^2 skin. For *Malassezia*, the lower limit of detection was 11 CFU/cm^2 skin. For the purposes of statistical analysis, samples from which no *Micrococcaceae* or propionibacteria were recovered were assigned 5·7 CFU/cm^2 skin, and where no *Malassezia* were recovered, a value of 1·1 CFU/cm^2 skin was assigned.

Differentiation of Malassezia furfur serovars A, B and C

Malassezia isolates were differentiated into serovars A, B and C according to the method of Cunningham *et al.*[10] Isolates with different colonial morphologies were emulsified in PBS, and 10 μl of the suspension air-dried on to each of the 12 wells of multispot slides. The slides were heat-fixed, then cooled, and 10 μl of the appropriate typing serum was applied to duplicate wells. The six typing sera were raised in rabbits against each serovar, using whole washed yeast cells. Each antiserum was absorbed with each heterologous yeast strain, i.e. (1) anti-serovar B absorbed with serovar A; (2) anti-serovar A absorbed with serovar C; (3) anti-serovar A absorbed with serovar B; (4) anti-serovar B absorbed with serovar C; (5) anti-serovar C absorbed with serovar B and (6) anti-serovar C absorbed with serovar A. Appropriate positive and negative controls were included. Slides were incubated at room temperature for 30 min, then washed in PBS, and 10 μl of anti-rabbit FITC-conjugated sheep

antiserum (Dako Ltd; diluted 1:70 in PBS) with Evans Blue (0·002% (w/v)) was applied to each well. Slides were incubated for a further 30 min at room temperature, and washed in PBS prior to examination using a Leitz fluorescent microscope (magnification ×500). Isolates were assigned to a serovar according to the following scheme. Serovar A gave positive reactions with antisera 2 and 3, serovar B with antisera 1 and 4, and serovar C with antisera 1, 3, 5 and 6.

Statistical analyses

Data were analysed by analysis of variance,[13] and results are expressed as the mean $\log_{10}$ CFU/cm^2 skin $\pm\frac{1}{2}$ minimum significant difference (MSD; calculated by the *T*-method). Means which differ by greater than the MSD ($P<0·05$) are significantly different at the 95% confidence level.

Results

The population densities of all organisms were expressed as the $\log_{10}$ CFU/cm^2. This was a reflection of the variation in the populations of skin commensals both between sites and between individuals.

Population densities of micro-organisms in pityriasis versicolor

The population densities of *M. furfur*, *Micrococcaceae* and propionibacteria on the chest, back, forehead and cheeks of patients with pityriasis versicolor and their age- and sex-matched controls are shown in Figure 1. The mean population density of *M. furfur* varied from 1·44 (patient forehead) to 3·56 $\log_{10}$ CFU/cm^2 (control back). There was no significant variation between the population density of *M. furfur* at a given site on the patients and the corresponding site on controls ($P>0·05$; 2-way analysis of variance [ANOVA]). However, there was a significant variation in the population densities of *M. furfur* between the five sites ($P<0·001$; 2-way ANOVA).

The mean population density of propionibacteria varied from 3·17 (patient chest) to 4·80 $\log_{10}$ CFU/cm^2 (control forehead). Analysis of the data showed that there was no significant variation between the population density of propionibacteria at a given site on the patients and the corresponding site on controls ($P>0·05$; 2-way ANOVA). No significant variation was found between the five sites for the population density of propionibacteria ($P>0·05$; 2-way ANOVA).

The mean population density of *Micrococcaceae* varied from 2·52 (patient back) to 4·66 $\log_{10}$ CFU/cm^2 (control cheek). There was no significant variation between the mean population density of *Micrococcaceae* at a given site on the patients and the corresponding site on controls ($P>0·05$; 2-way ANOVA), but there was a significant variation ($P<0·001$; 2-way ANOVA) between the five sites sampled.

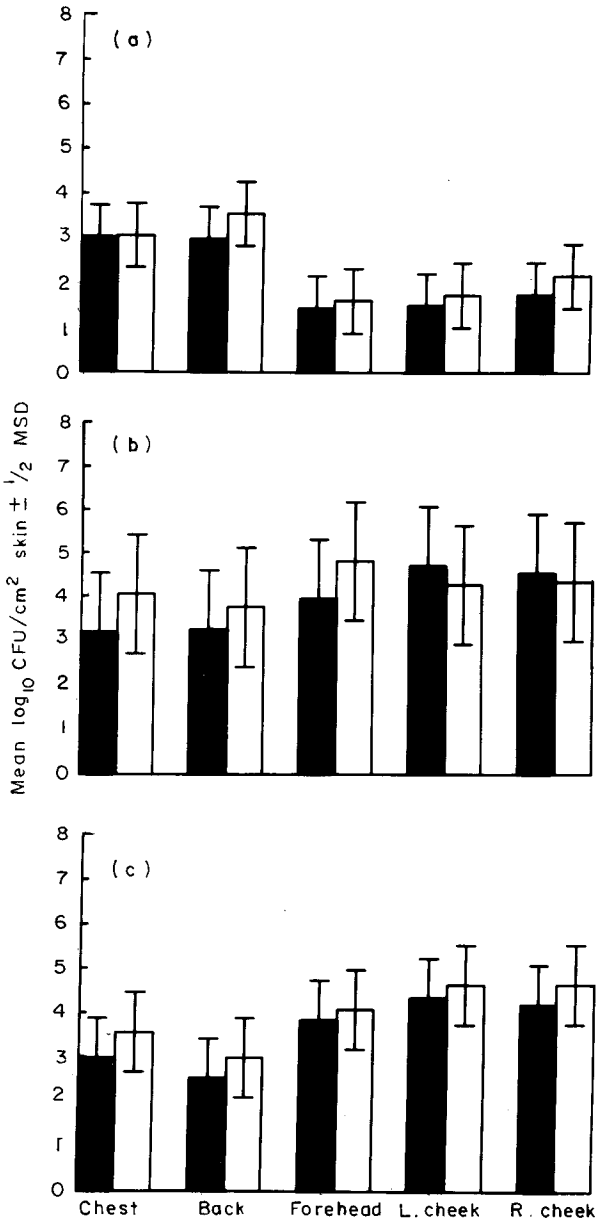

Figure 1. The population density of (a) *Malassezia furfur*, (b) propionibacteria and (c) *Micrococcaceae* on pityriasis versicolor patients, and age- and sex-matched controls, on the chest, back, forehead and left and right cheeks. Results are expressed as the mean $\log_{10}$ CFU/cm^2 skin ($n=10$). Data was analysed by 3 × 2-way ANOVA. Error bars represent the minimum significant difference calculated by the *T*-method. Error bars which do not overlap are significantly different at $P<0·05$. ■, pityriasis versicolor patients; □, controls.

Population densities of Malassezia furfur serovars A, B and C in pityriasis versicolor

Malassezia furfur isolates were differentiated into serovars A, B and C. The mean $\log_{10}$ CFU/cm^2 skin of the three serovars on the chest, back, forehead, left and right cheeks of patients with pityriasis versicolor and controls is shown in Figure 2. Serovar A was the predominant isolate on both the chest and back, with a mean $\log_{10}$ CFU/cm^2 skin of 2·84–3·53 compared with 0·04–0·62 for serovars B and C ($P<0·001$; 3-way ANOVA). On the forehead and cheeks there was no significant difference between the mean population densities of the three serovars when tested using the MSD. There was a significantly greater density of serovar A on the chest and back than on the head regions when tested using the MSD, but the population densities of serovars B and C did not vary between sites.

When patients were compared with controls there were no differences between the population densities of serovars A, B and C at any of the five sites, when compared using the MSD. All the pityriasis versicolor patients had lesions on the chest. Thus, this data indicated that there was no difference in either the total population density of *M. furfur*, or the distribution of serovars, on lesional skin, compared with control skin at this site.

Population densities of micro-organisms in seborrhoeic dermatitis

The population densities of *M. furfur*, propionibacteria and *Micrococcaceae* on the chest, back, forehead and cheeks of patients with seborrhoeic dermatitis and their age- and sex-matched controls are shown in Figure 3. The mean population density of *M. furfur* varied from 1·73 (control forehead) to 3·23 $\log_{10}$ CFU/cm^2 (control chest). There was no significant variation between the population density of *M. furfur* at any given site on the patient and the same site on the control ($P>0·05$; 2-way ANOVA). A significant variation was found in the population density of *M. furfur* between the five sites ($P<0·001$; 2-way ANOVA).

The mean population density of propionibacteria varied from 3·86 (patient back) to 5·44 $\log_{10}$ CFU/cm^2 (patient cheek). The population density did not vary significantly between patients and controls at any given site ($P>0·05$; 2-way ANOVA). There was also no significant variation in the population density of propionibacteria between the five sites ($P>0·05$; 2-way ANOVA).

The mean population density of *Micrococcaceae* varied from 2·80 (patient back) to 4·84 $\log_{10}$ CFU/cm^2 (patient

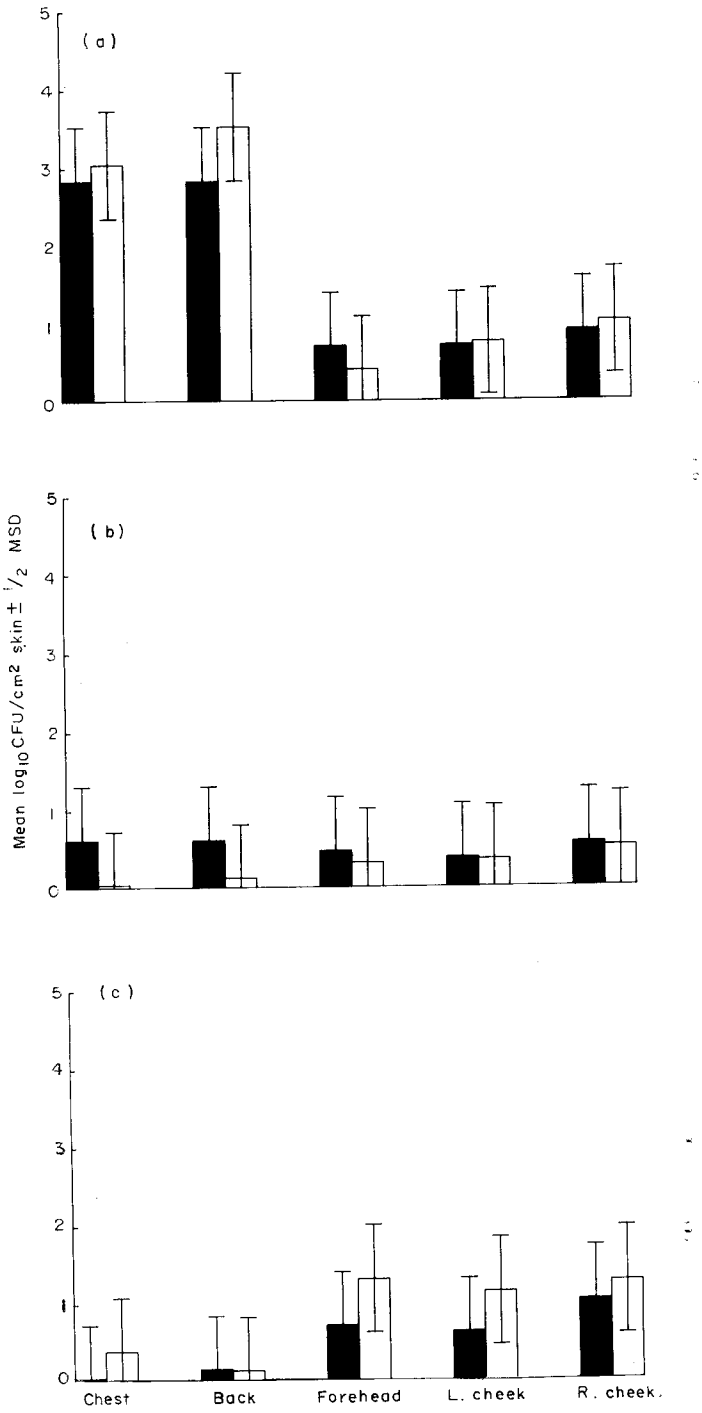

Figure 2. The population density for *Malassezia furfur* serovars A (a), B (b) and C (c) on pityriasis versicolor patients, and age- and sex-matched controls, on the chest, back, forehead, left and right cheeks. Results are expressed as the mean $\log_{10}$ CFU/cm^2 skin ($n=10$). Data was analysed by 3-way ANOVA. Error bars represent the minimum significant difference calculated by the *T*-method. Error bars which do not overlap are significantly different at $P<0·05$. ■, pityriasis versicolor patients; □, controls.

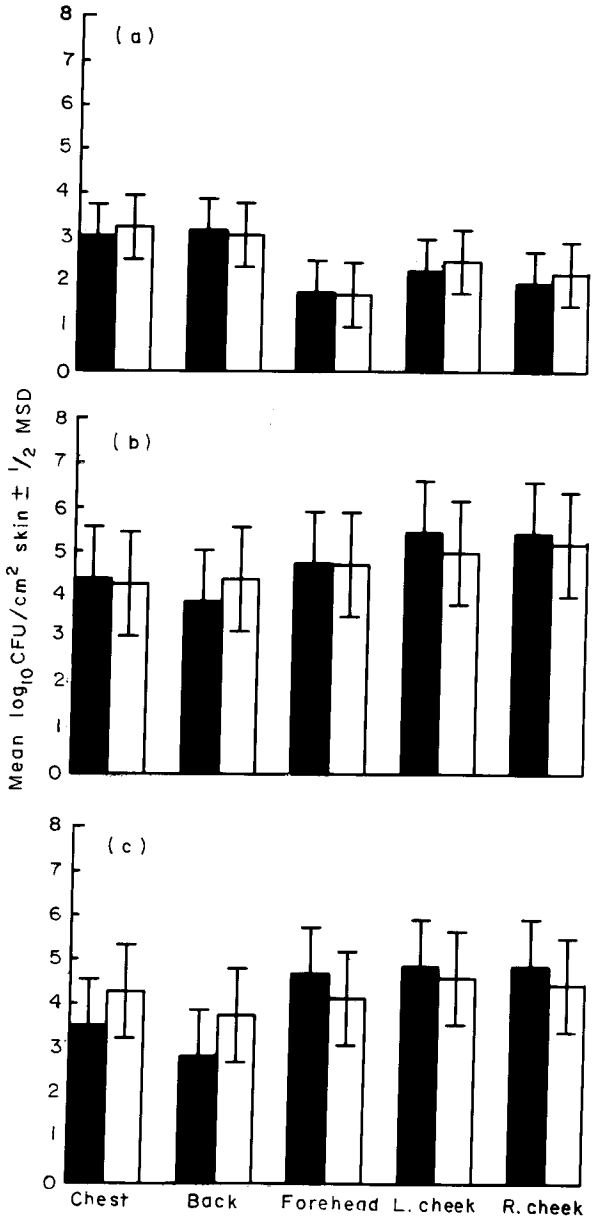

Figure 3. The population density of (a) *Malassezia furfur*, (b) propionibacteria and (c) *Micrococcaceae* on seborrhoeic dermatitis patients, and age- and sex-matched controls, on the chest, back, forehead and left and right cheeks. Results are expressed as the mean $\log_{10}$ CFU/cm^2 skin ($n=10$). Data was analysed by 3 × 2-way ANOVA. Error bars represent the minimum significant difference calculated by the *T*-method. Error bars which do not overlap are significantly different at $P<0{\cdot}05$. ■, seborrhoeic dermatitis patients; □, controls.

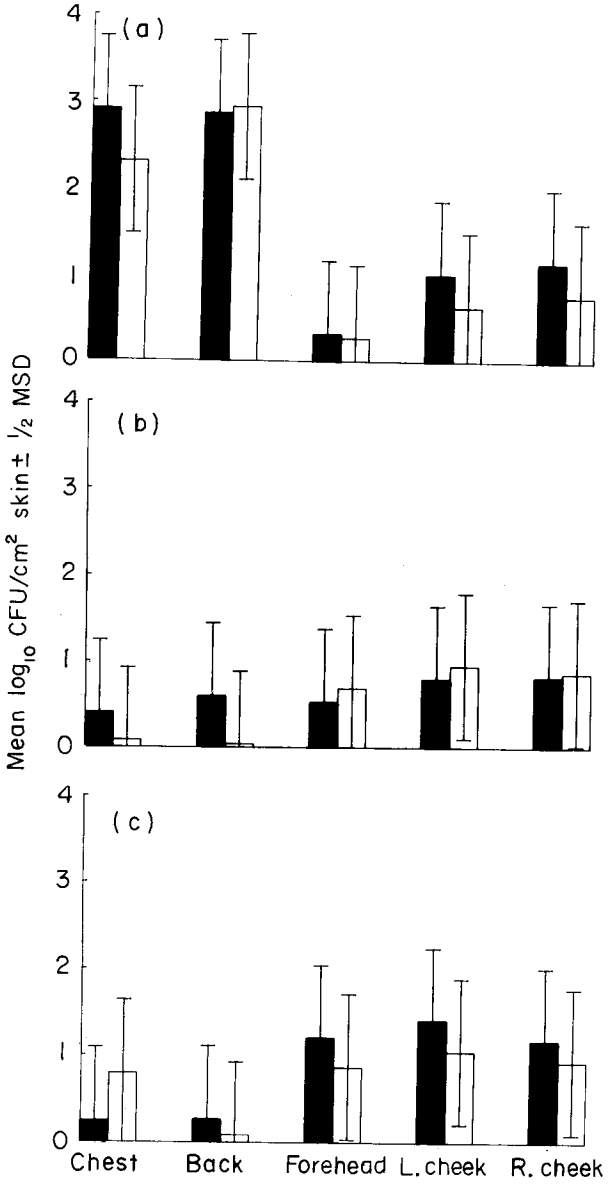

Figure 4. The population density for *Malassezia furfur* serovars A (a), B (b) and C (c) on seborrhoeic dermatitis patients, and age- and sex-matched controls, on the chest, back, forehead, left and right cheeks. Results are expressed as the mean $\log_{10}$ CFU/cm^2 skin ($n=10$). Data was analysed by 3-way ANOVA. Error bars represent the minimum significant difference calculated by the *T*-method. Error bars which do not overlap are significantly different at $P<0{\cdot}05$. ■, seborrhoeic dermatitis patients; □, controls.

cheek). There was no significant variation in the population density of *Micrococcaceae* between the patients and controls at a given site, but there was a significant variation ($P<0{\cdot}025$; 2-way ANOVA) between the five sites.

Population densities of Malassezia furfur serovars A, B and C in seborrhoeic dermatitis

The mean $\log_{10}$ CFU/cm^2 skin of the three serovars on the chest, back, forehead and cheeks of patients with seborrhoeic dermatitis are shown in Figure 4. Serovar A was the predominant isolate on both the chest and back,

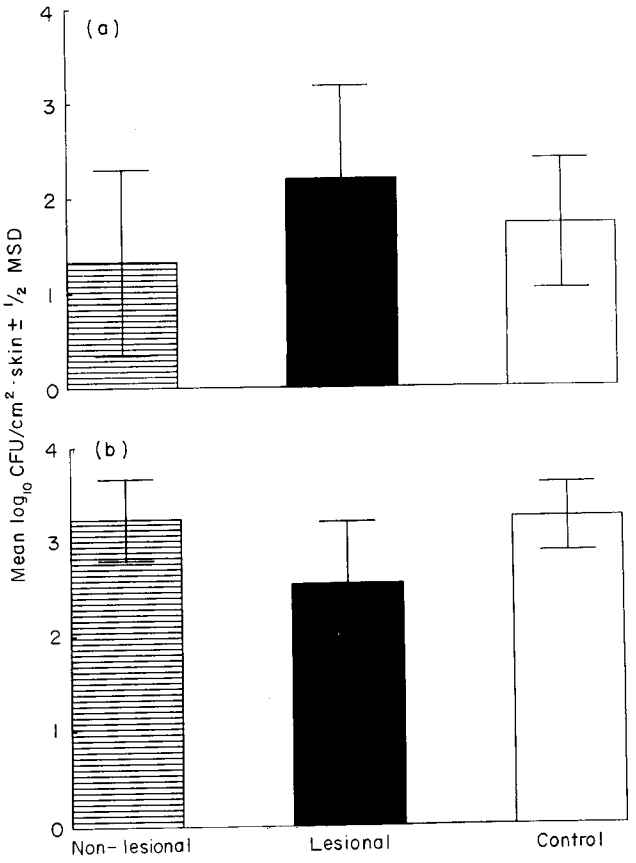

Figure 5. The population density of *Malassezia furfur* on seborrhoeic dermatitis patients, and age- and sex-matched controls, on the (a) forehead and (b) chest, on lesional, non-lesional and control skin. Results are expressed as the mean $\log_{10}$ CFU/cm^2 skin. Data was analysed by 2-way ANOVA. Error bars represent the minimum significant difference calculated by the *T*-method. Error bars which do not overlap are significantly different at $P<0{\cdot}05$. ■, lesional; ▤, non-lesional; □, control.

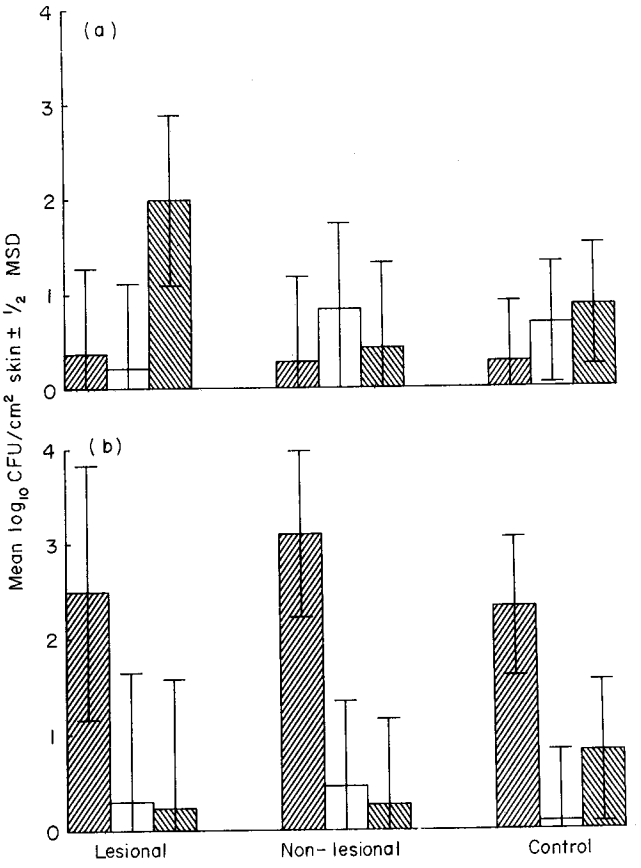

Figure 6. The population densities of *Malassezia furfur* serovars A, B and C on seborrhoeic dermatitis patients, and age- and sex-matched controls, for the (a) forehead and (b) chest, on lesional, non-lesional and control skin. Results are expressed as the mean $\log_{10}$ CFU/cm^2 skin. Data was analysed by 2-way ANOVA. Error bars represent the minimum significant difference calculated by the *T*-method. Error bars which do not overlap are significantly different at $P<0{\cdot}05$. ▨, A; □, B; ▧, C.

with a mean $\log_{10}$ CFU/cm^2 skin of 2·86–2·91 compared with 0·25–0·60 for serovars B and C (MSD = 1·68). In contrast with this, the mean population densities of serovars B and C did not differ significantly between sites. On the forehead and cheeks there was no difference between the mean population densities of the three serovars, when tested using the MSD. There was no difference at any site when the mean population density on patients was compared, using the MSD, with that on controls.

The distribution of lesions on patients with seborrhoeic dermatitis was very different from that of the patients with pityriasis versicolor. Of the 10 patients with seborrhoeic dermatitis, three had lesions on the chest and five had lesions on the forehead. Because of this, it was possible to compare the carriage of the three serovars of *M. furfur* at lesional and non-lesional sites. The mean $\log_{10}$ CFU/cm^2 skin of *M. furfur* for lesional, non-lesional and control skin on the chest and forehead of seborrhoeic dermatitis patients are shown in Figure 5. There was no difference in the mean total population density of *M. furfur* for the lesional, non-lesional and control skin, when compared using the MSD. The mean $\log_{10}$ CFU/cm^2 skin for each of the three serovars of *M. furfur* for the lesional, non-lesional and control sites on the chest and forehead of seborrhoeic dermatitis patients and controls are shown in Figure 6. On the non-lesional and control chest sites, the mean $\log_{10}$ CFU/cm^2 skin of serovar A was significantly higher than that of serovars B and C, when compared using the MSD. However, on the lesional chest sites there was no significant difference between the mean population densities of any of the serovars. On the forehead sites, the mean population density of the serovars did not differ significantly

between the lesional, non-lesional and control sites. However, although it did not reach statistical significance, the population density of serovar C on lesional skin was 10-fold greater than on non-lesional and control skin.

Discussion

The aim of this study was to establish whether there was any association between the newly defined serovars of *M. furfur* and the lesional skin of patients with pityriasis versicolor or seborrhoeic dermatitis. For this purpose, the study was designed to gain an accurate quantification of the population densities of the serovars of *M. furfur* on both the lesional and non-lesional skin of patients, compared with age- and sex-matched controls, using a limited number of individuals. The study clearly demonstrated that the lesional skin of patients with pityriasis versicolor and seborrhoeic dermatitis harbours *M. furfur* serovars A, B and C, and that the numbers of the serovars on lesional skin do not differ significantly from (a) non-lesional skin at the same sites, or (b) control skin. Thus, there was no evidence that any particular serovar of *M. furfur* or combination of serovars is associated with either disease.

The population densities of *M. furfur*, propionibacteria and *Micrococcaceae* varied widely between patients and controls. However, such variation is part of natural biological variation, and as such it is not possible to overcome it. In order to gain an idea of the population densities for each group as a whole, the results presented here are the mean values for that group.

Leeming *et al.*[4] showed that the population density of *M. furfur* was highest on those areas of the body which have the greatest density of sebaceous glands. These areas are also the sites which are most frequently affected by *Malassezia*-associated dermatoses. The sites sampled in this study reflected the known distribution of *Malassezia* and the lesions of pityriasis versicolor and seborrhoeic dermatitis. Throughout the study it was found that the distribution of the three serovars of *M. furfur* differed between body sites. On the chest and back serovar A predominated, whereas on the forehead and cheeks no one serovar was predominant. This distribution was found in all four groups, whether or not the site was involved in either disease. This differential distribution of the serovars was first noted by Leeming and Notman,[7] but they did not study it in depth, and later by Cunningham *et al.*[10] The reasons for this difference in the distribution of the serovars may be related to their different physiological requirements. Cunningham *et al.*[10] found that serovar A was nutritionally less fastidious, and reached a higher yield *in vitro* than either serovars B or C. If this difference also occurred *in vivo* it would enable serovar A to reach a higher population density on less favourable areas of the skin. However, certain areas of the skin could also be less favourable due to the presence of inhibitory substances.

Several authors have examined the carriage of *Malassezia furfur* in patients with pityriasis versicolor or seborrhoeic dermatitis. There has been controversy as to whether there is an increase in the number of organisms on lesional skin, and thus, whether overgrowth alone could be responsible for the development of the diseases. The studies of McGinley *et al.*,[14] Clift *et al.*,[15] and Bergbrant and Faergemann[16] did not find any difference in the carriage of *M. furfur* on seborrhoeic dermatitis-affected skin compared with control skin. McGinley *et al.*[14] classified seborrhoeic dermatitis and dandruff separately, and found that patients with dandruff had a significantly greater density of *M. furfur* on the scalp than controls, but not greater than seborrhoeic dermatitis patients. In contrast, Heng *et al.*[17] found that not only did seborrhoeic dermatitis-affected sites on the face and scalp have significantly greater densities of *M. furfur* than control skin, but that the number of organisms was related to the severity of the disease. They also found that after treatment with antifungal drugs, the density of *M. furfur* decreased parallel to the clinical response. Heng *et al.*[17] performed total counts on the samples, but did not attempt to culture *M. furfur*. This method may lead to inaccuracies, because cells may be obscured from view by debris, or debris may be included as yeast cells. The differences observed in carriage could be explained, at least in part, by differences in the ages of the groups, as *M. furfur* carriage varies with age. Heng *et al.*[17] stated that their controls were age-and sex-matched, but they included 42 controls and 100 patients. In 1970, McGinley *et al.*[18] found, in their study of the microbiology of pityriasis versicolor, that lesional sites had many more yeast and mycelial elements, as measured by total counts, than non-lesional sites on the same patients. However, they did not comment on whether these differences reached statistical significance. Cells included in total counts may not be viable, and thus, total counts and viable counts are not directly comparable. The relative importance of the yeast and mycelial phases in the development of pityriasis versicolor and seborrhoeic dermatitis is still a matter of controversy. During this present study, samples were not examined for the presence of mycelial elements, and this raises the possibility that what has been measured is not an

accurate reflection of the situation on the skin. However, there is no medium which is able to select for mycelia and suppress the growth of the yeast phase of *M. furfur*. Attempts to count mycelial elements are also problematic, as mycelia may be of different lengths but still constitute a single mycelium. The measurement of biomass would overcome this problem.

The population densities for *Micrococcaceae* and propionibacteria did not differ significantly between patients and controls for any site sampled. This contrasts with the results of McGinley *et al.*,[18] who found that in a group of 31 patients with pityriasis versicolor, the population density of the aerobic flora was significantly higher on lesional than non-lesional skin. McGinley *et al.*[14] sampled 63 patients with seborrhoeic dermatitis and 112 controls. They found that the density of propionibacteria was significantly lower on seborrhoeic dermatitis-affected scalp sites than on control scalp sites, but that there was no difference in the aerobic flora. However, the controls used were not matched according to age and sex, and thus the differences observed may have been partly due to the differences in carriage at different ages rather than to seborrhoeic dermatitis.

In summary, this study did not find any difference in the total population density of *M. furfur* in patients with either pityriasis versicolor or seborrhoeic dermatitis, when compared with age- and sex-matched controls. Nor was there any significant difference between lesional and non-lesional sites. This supports the theory that although *M. furfur* may be the aetiological agent of pityriasis versicolor and seborrhoeic dermatitis, there are factors other than a simple overgrowth of a particular strain of *M. furfur* which contribute to the development of the dermatoses.

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