ELISA Subtypization of Anti-ENA Autoantibodies in Clinical Management of Autoimmune Diseases in Bosnia and Herzegovina

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SUMMARY
The basis of autoimmune diseases such as SLE (Systemic Lupus Eritematosus), Sjögren’s syndrome, scleroderma, dermatomyositis and polymyositis is the creation of auto-antibodies to the following specific extractable nuclear antigens (ENA): Jo-1, Scl-70, SS-A, SS-B, Sm and Sm/RNPs. Some of these antigens are in fact enzymes (Jo-1–histidyl-tRNA synthetase, Scl-70–topoisomerase) which are inhibited by specific autoantibodies - this leads to disturbance in the metabolism of DNA and protein biosynthesis. During 2009, we analyzed total of 87 serum samples of patients suspected for autoimmune disorder using ANA-IFA and ELISA-ENA-6 methods. After establishing IFA-ANA positivity (83,9%), all serum specimens, ANA positive and negative, were subtypized by ELISA-ENA-6 test. Analysis showed the highest incidence of anti-SS-A (56,0%), and incidence of anti-SS-B (29,8%), anti-Sm/RNP (11,5%), anti-Jo-1 (2,3%) and anti-Scl-70 (1,1%) auto-antibodies. Also, 78,5% of IFA-ANA negative serum specimens showed high level of positivity (212,50–277,0 U/ml) to SS-A (78,5%) and SS-B (21,4%) antigens using ELISA-ENA-6 subtypization. Following these results, we conclude that it is necessary to introduce Western blot confirmation testing. After comparing with other clinical findings, we diagnosed the following autoimmune diseases: SLE, Sjögren’s syndrome and dermatomyositis.

Keywords: autoimmunity, IFA-ANA, ELISA-ENA-6, serum specimens, subtypization

1. INTRODUCTION
The presence of antinuclear antibodies (ANA) is a hallmark of many systemic autoimmune diseases (AID). ANAs are especially found in active and inactive systemic lupus erythematosus (SLE), mixed connective tissue diseases (MCTD), scleroderma, Sjögren’s syndrome, and polymyositis.

The technique commonly used for their detection is indirect immunofluorescence assay (IFA) with fixed tissue or cultured cell lines as the substrate. Moreover, it is necessary to detect auto-antibody reactivity against specific extractable nuclear and cytoplasmic antigens (ENA) such as:

- **Sm (Smith antigen)** – The core proteins of small nuclear ribonucleoproteins. Anti-Sm are specific for SLE. These proteins with (sn-RNPs) or small nuclear ribonucleoprotein particles makes together specific complex, important for processing of pre-mRNAs molecules.
- **snRNP/Sm complex** – They occur in SLE, Sjögren’s syndrome, scleroderma and polymyositis.
- **SS-A (Ro)** – (soluble cytoplasmic and/or nuclear ribonucleoproteins of 52 kDa and 60 kDa molecular weight) specific for Sjögren’s syndrome.
- **SS-B (La)** – (48 kDa molecular weight protein, associated with RNA polymerase III). Anti-SS-B autoantibodies are mainly found in high titers for primary and secondary Sjögren’s syndrome, but also in SLE and neonatal lupus.
- **Scl-70** – (DNA-topoisomerase I). Anti-Scl-70 antibodies are highly specific for systemic scleroderma.
- **Jo-1** – (histidyl-tRNA synthetase-cytoplasmic protein involved in protein biosynthesis). Anti-Jo-1 antibodies are found in 20–40 % of patients with polymyositis and dermatomyositis.

The antinuclear antibodies were first discovered 50 years before and found to be associated with connective tissue diseases (CTDs). Since then, different methods have been described and used for their detection or confirmation. For many decades, immunofluorescent antinuclear antibody test, has been the “gold standard” in the diagnosis of these disorders. The intensity of fluorescence patterns, are directly proportional to antinuclear antibody (ANA) concentration and expressed with a quantitative scale of values. Its evaluation is crucial as low titer is less significant than a high titer and may be seen even in healthy individuals. There are many studies which have attempted to determine the optimum screening dilution of sera for ANA testing. A titer of 1:160 is taken as significant for the diagnosis of CTDs in majority of laboratories (1, 2, 3, 4, 5).

Although IFA-ANA test is widely used and considered to be gold standard, still the results may sometimes be misinterpreted. As it detects several different antibodies cross-reactions can occur. In up to 3% of the normal population it can give false positive result. Certain patterns i.e. nucleolar and centromeric are less well defined by IFA-ANA tests. The test therefore is mainly used for screening, rather than to diagnose a CTD (6, 7, 8).

There are two types of ELISA or ELISA methods currently used for ANA testing. One is called generic assay which detects ANA of broad specificity similar to IFA-ANA. The other is antigen specific assay that detects ANA and reacts with a single autoantigen i.e. dsDNA, SS-A/Ro, SS-B/La, Scl-70, Sml, Sm/RNP, Jo-1. In the 87-95% cases IFA-ANA and ELISA-ENA-6 show very similar results. The sensitivity of the various ELISAs was 69% to 98% and the specificity ranged between 81% and 98% (9, 10).

In our investigations we performed the ELISA-ENA-6 subtypization of all 87 IFA-ANA positive and negative serum specimens, taken from patients suspected to autoimmune diseases.

2. MATERIAL AND METHODS
IFA-ANA TEST
We used NOVA Lite HEP-2 immunofluorescence kit for screening and semi-quantitative determination of antinuclear antibodies (ANA) in serum specimens. The kit contains the slides with HEP-2 cells as substrate, FITC (fluorescin isothiocyanate incorporated into anti-human IgG), ready to use positive control – human serum, ready to use negative control-human serum, PBS (phosphate saline buffer), ready to use mounting medium (glycerine in phos-
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PBS buffer is diluted with distilled water to final 1x concentration. Serum specimens were diluted with PBS buffer.

It was added one drop (25µl) of diluted serum specimens and controls as well, into slide wells. After incubation and rinsing procedures, the IgG FITC-EVANS was added and on the end, several drops of Mounting medium.

The slides were examined by fluorescence microscope (495 nm excitation filter and a 525 nm emission filter). For obtaining of best results the slides are analysed immediately.

Immunofluorescence determination of anti-nuclear antibodies has been accepted as the most suitable method for identification or confirmation of connective tissue diseases, such as SLE (99%), sclerodermia (73%), Sjögren’s disease (50%), early rheumatoid arthritis (20%), auto-immune chronic hepatitis (50-60%), primary biliary cirrhosis (35-40%), and cryptogenic cirrhosis (45%).

Fluorescence intensity may be semi-quantified by following the guidelines established by the Center for Diseases Control, Atlanta, Georgia.

- 4 (++) Top fluorescence: bright apple-green.
- 3 (++) Light-up apple-green fluorescence.
- 2 (+) Distinctly clear apple-green fluorescence.
- 1 (+) Opaque yet visible apple-green fluorescence.
- 0 (-) Non-fluorescent or lacking any visible fluorescence.

Regarding to type of fluorescence, the following fluorescence patterns can be found: homogenous, peripheral, speckled and nucleolar. Each of them represent specific marker in diagnostic process of autoimmune diseases (11, 12, 13, 14).

ELISA-ENA – 6 TEST

ENA (Extractable nuclear antigens)-6 Profile ELISA is a solid phase enzyme immunoassay for the separate semi-quantitative detection of IgG antibodies against six cellular and nuclear antigens in human serum.

The wells are coated with recombinant SS-B, SS-A 52 kDa, Scl 70, Jo-1 and highly purified native human snRNP/
Sm, Sm and SS-A 60 kDa antibodies. The assay is a tool in the differential diagnosis of systemic rheumatic diseases.

Into antigen precoated ELISA microplate wells (SS-A, SS-B, Sm, Sm/RNP, Jo-1, Scl-70) it were added 100µl of controls, calibrators and diluted (1:101) serum specimens. After incubation at room temperature, first washing with 300µl washing buffer per well, it was pipetted 100µl of conjugate (anti-human IgG antibody conjugated with horseradish peroxidase as enzyme) into each well and incubated at room temperature 30-35 minutes.

After second washing procedure, it was added into wells chromogen substrate. Duration of incubation in the dark has been 10 minutes followed by 30 minutes at 450, 405, 620 and 690 nm of wavelengths. The obtained absorbance values are expressed in IU/ml.

We performed the ELISA-ENA-6 subtypisation by fully automated HYTEC™ 288 Plus System. HYTEC 288 Plus features are: high-precision, robotic liquid handling, real-time incubation control and on-board quality control analysis to ensure test accuracy. HYTEC 288 Plus is compatible with many interface protocols and provides quality control management. It complies with FDA Quality System Regulation, current International ISO Standards, NCCLS guidelines and CE-mark guidelines (15, 16, 17, 18).

3. RESULTS

Total 87 serum specimens, taken from clinical patients suspected to autoimmune diseases, were analysed by indirect immunofluorescence assay (IFA-ANA) to presence of antinuclear antibodies. Positive results showed 73 of them or 83,9% which means unusually very high their prevalence. 2+ fluorescence intensity have had 18 samples and others 1+ degree positivity. The prevalence of ANA negative samples has been 16,1% (14 serum samples).

In the second step of our investigation, we analysed by ELISA-ENA-6 assay both, IFA-ANA positive and IFA-ANA negative serums. Most of ANA negative samples (11 from 14 or 78,5%) showed the presence of specific autoantibodies especially to SS-A (78,5%) and anti-Sm/RNP (10 or 11,5%) antibodies. Also, only one serum (1,1%) have had autoantibodies to Jo-1 and Scl-70 autoantigens.

The estimated total prevalences of specific ENA-6 autoantibodies after analyses of both positive and negative ANA-IFA specimens were:

- SS-A – 49 or 53%
- SS-B – 26 or 28%
- Jo-1 – 2 or 2%
- Sm – 5 or 5%
- Sm/RNP – 10 or 11%
- Scl-70 – 1 or 1%

4. DISCUSSION

The basis of autoimmune diseases such as SLE (Systemic Lupus eritematoses), Sjogren’s syndrome, scleroderma, dermatomyositis, polymyositis is creating autoantibodies to the following specific extractible nuclear antigens (ENA): Jo-1, Scl-70, SS-A, SS-B, Sm and Sm/RNPs. Some of these antigens are in fact enzymes (Jo-1–histidyl-tRNA synthetase, Scl-70–topoisomerase). In the specific pathological conditions, newly synthesized autoantibodies inhibit them. On this way, the normal flow of DNA metabolism and protein biosynthesis is disturbed. The

<table>
<thead>
<tr>
<th>Spec. No.</th>
<th>ANA-ENA Negative</th>
<th>ELISA SS-A IU/ml</th>
<th>ELISA SS-B IU/ml</th>
<th>ELISA Sm IU/ml</th>
<th>ELISA Sm/RNP IU/ml</th>
<th>ELISA JU/ml</th>
<th>ELISA Scl-70 IU/ml</th>
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<td>1</td>
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<tr>
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<td>3.28</td>
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<td>2.24</td>
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Sm/RNPs antigens play a key role in the splicing reactions (introns removing and exons junction), in the process of pre-mRNA posttranscriptional processing and creation of a mature mRNA molecule. SS-A antigens bind to small RNA molecules labeled HY-Rnase. These complexes have been designated as Ro-RNP and their biological function has not yet been clarified. It is believed that the SS-B antigen is an helicase enzyme. It has the ability to bind the oligo (U) 3’ end of RNA nascent transcripts, accumulated by of enzyme polymerase III activity, and participates in the regulation of process transcription termination and processing of primary RNA transcripts (19, 20, 21).

The incidence of anti-Ro (SS-A) antibodies is 1%, in the population of normal women of reproductive age. The presence of anti-La (SS-B) autoantibodies usually coincides with the presence of anti-Ro (SS-A).

Autoantibodies to the Ro (SS-A) antigen are one of the most frequent serological markers of autoimmune in rheumatic diseases. They are present in the serum of 50-80% of patients with Sjögren’s syndrome (SS), 30-40% of patients with systemic lupus erythematosus (SLE), and 3-5% of patients with rheumatoid arthritis (RA). Ro (SS-A) autoantibodies have also been reported to be present in Sjögren’s syndrome, lupus overlap disease, subacute cutaneous lupus erythematosus, lupus with complement component deficiencies, neonatal lupus syndrome, multiple myeloma, polymyositis, progressive systemic sclerosis and primary biliary cirrhosis.

Our results showed the highest prevalence of anti-SS-A antibodies (56%).

La (SS-B) antigen binds to the oligo(U) 3’ termini of nascent RNA polymerase III transcripts and facilitates transcriptional termination and reinitiation by this enzyme. It has also been reported to function as an ATP-dependent helicase able to melt RNA-DNA hybrids. Sjögren’s syndrome (SS) is a common systemic auto-immune inflammatory disorder characterised by lymphocyte-mediated destruction of exocrine glands leading to diminished or absent glandular secretion. SS may present as a primary disease or in association with other systemic autoimmune diseases.

Anti-Scl-70 is recognized as one of two major classes of autoantibodies in sclerosis (systemic or scleroderma). The antigen of Anti Scl-70 was recognized as topoisomerase I in 1986 with anti-centromere antibodies constituting the other class. ATA is associated with more severe disease. Anti-topoisomerase antibodies (ATA) can be classified according to their immunoglobulin class (IgM, IgG or IgA). IgG-ATA is found most frequently in scleroderma, with IgA being quite common but IgM very infrequent. Topoisomerase I is an enzyme that relaxes the strain on DNA by nicking and ligating the DNA. ATA inhibits the activity of this enzyme. Since this activity occurs in the nucleus of the cell ATA is a form of anti-nuclear antibody. Scleroderma results from the overproduction of collagen in affected tissues, one study claims that there is an increased density of Topoisomerase I in the collagen genes, and that the antibodies may be altering transcription at these loci. ATA correlates with rapid progression of disease.

Anti-Jo-1 antigen. This enzyme belongs to the family of ligases specifically those forming carbon-oxygen bonds in aminoacyl-tRNA and related compounds. The systematic name of this enzyme class is \textbf{L-histidyl-tRNA synthetase}, which catalyzes the formation of L-histidyl-tRNA, a key intermediate in the translation process. Anti-Jo-1 antibodies are found in patients with myositis, especially those with lung involvement (Medsgen and Oddis 1994). Anti-Jo-1 antibody recognizes histidyl-tRNA synthetase (Jo-1), a dimer of 50 kDa subunits found in the cytoplasm that participates in histidine metabolism and aminoacyl-tRNA biosynthesis. Autoantibodies to histidyl-tRNA synthetase (HisRS) or to alanyl–, aspartyl–, glycy–, isoleucyl–, or threonyl–tRNA synthetase occur in 25% of patients with polymyositis or dermatomyositis. However, the specificity of such autoantibodies has to be evaluated in daily clinical practice. It is necessary to search for anti-Jo1 autoantibodies even if antinuclear antibodies are neg...
ative by indirect immunofluorescence and underlines the usefulness of anti-Jo1 antibodies of titer above 60 AU/mL in the diagnosis of complete or incomplete ASS (antisynthetase syndrome) (29,30,31). Detected ant-Jo-1 antibodies prevalence was 2,3%.

By analysis with other clinical findings, it were diagnosed the following autoimmune diseases: dermatomyositis, SLE, Sjögren’s syndrome, and scleroderma in one patient.

It would be interesting, in the next stage of the research, to compare the obtained results with the appropriate data from before the war in relation to stress and UV radiation factors that are reliably known to have large impact on the occurrence of autoimmune diseases.

Because of determined very high positivity after both IFA-ANA and ELISA-ENA-6 testing, it must be introduced confirmative western blot analyses.

Besides this aspect of epidemiological research, it is necessary to explore the molecular genetic basis, particularly HLA-I, HLA-II, IRF-5 and the Bank-1 gene in cases of SLE and in the case dermatomyositis and polymyositis the polymorphism of HLA-DR3 gene locus. This would certainly supplementing molecular-epidemiological aspects of autoimmune diseases in BiH.

5. CONCLUSIONS

- The incidence of IFA-ANA positivity regarding to specimens number was very high – 83,9 %.
- Almost all positive specimens showed both SS-A and SS-B positivity.
- Neither specimen did not show the presence of autoantibodies to all ENA specific antigens together.
- ANA subtypisation made by ELISA ENA-6 test showed the highest incidence of anti-SS-A (44,8 %) and anti-SS-B (27,5 %).
- The prevalence of autoantibodies to other nuclear antigens (Sm, Sm/RNP, Jo-1, Scl-70) ranged from 1,1 % (Jo-1) and Scl-70 to 5,7% for Sm and 11,5% for Sm / RNP.
- ELISA anti-SS-A positivity expressed in IU / ml, yet almost always been accompanied with somewhat lower values of SS-B positivity.
- Only three samples showed ENA-6 values (IU / ml) near the border of absolute positivity.

Two have had positive values for SS-A, SS-B, Sm and Sm/RNP and one for SS-B, Sm/ RNP, Jo-1 and Scl-70. Other negative values were close to positive border.

- According to the our obtained results and other clinical finding as well, it has been diagnosed following autoimmune diseases: Sjögren’s syndrome, SLE and dermatomyositis.
- It is necessary to do, with SLE diagnosed cases, additional analysis to the presence of anti-histone antibodies, to determine whether the illness caused by drugs.
- It would be interesting, do the additional confirmative Western blot analysis of SLE diagnosed cases.
- As for illuminating the molecular basis of genetic origin of these autoimmune diseases, it would be useful to analyze the special cases outlined in the polymorphism of HLA-I, HLA-II, IRF-5 and the Bank-1 gene, because there is a possibility of correlation of specific genetic predisposition to the appearance of SLE.
- In the cases polymyositis and dermatomyositis, is necessary to analyze the HLA-DR3 polymorphism on the molecular level.

REFERENCES

1. INTRODUCTION

Lipophilic yeasts of the genus Malassezia (former Pityrosporum) are members of the normal cutaneous microflora of humans and other warm-blooded animals. However, under the influence of predisposing factors these yeasts can become agents of pityriasis versicolor (PV) or associated with seborrhoeic dermatitis (SD) and psoriasis (PS) and even systemic infections (1, 2).

In 1996, the taxonomy of the genus Malassezia was enlarged to include seven species, namely M. furfur, M. pachydermatis, M. sympodialis, M. slooffiae, M. globosa, M. obtusa and M. restricta (3). Furthermore, in last few years some new species have been isolated from human (M. dermatis, M. japonica and M. yamotensis) and animal skin (M. nana, M. caprae and M. equina) (4, 5, 6, 7, 8). However, these species have been identified using ribosomal DNA sequence analysis and not with a cultivation identification system. Therefore, further biochemical and molecular characterization are required for their acceptance as distinct species.

M. pachydermatis is unique within the genus is that is non lipid-dependent and the most common species adapted to animals, in particular to domestic and wild carnivores (9, 10). This fungus occurs rarely on humans, although it has been found to cause intravascular catheter-acquired infections in premature neonates and, less frequently, in immunocompromised adults (1, 11). In contrast, the lipid-dependent species known to be mainly associated with humans, can also be isolated from animals (7, 8, 12, 13).

So far, few studies have been published regarding the distribution of M. pachydermatis on human skin. Therefore, the aim of this study was to analyze the prevalence of M. pachydermatis on the skin of patients with Malassezia-related skin diseases such as PV, SD and PS on normal skin.

2. PATIENTS AND METHODS

The group studied included 40 patients with PV (24 female and 16 male; 16-67 years old), 40 patients with SD (17 female and 23 male; 7-71 years old), and 40 patients with PS (18 female and 22 male; 7-72 years old). Normal subjects consisted of 40 healthy volunteers (20 female and 20 male; 13-76 years old) without any evidence of a dermatosis. Only those subjects who had not used any topical and oral treatment in previous two months were included in the study. All samples consisted of skin scales and scrapings: in PV patients from the lesional skin of the trunk, in SD and PS patients from the scalp lesions and in healthy subjects from the upper part of the trunk. All participants gave their informed consent with the requirements of the Institutional Ethics Committee.

The scales were inoculated into Sabouraud dextrose agar and into modified Dixon agar consisting of 3.6% malt extract, 0.6% mycological peptone, 2.0% desiccated ox bile (Sigma Chemical Co. Ltd, Dorset, UK), 1% Tween 40, 0.2% glycerol, 0.2% oleic acid, 0.05% chloramphenicol, 0.05% cycloheximide, and 1.2% agar pH 6.0. The medium was always used within one week of preparation and the cultures were inoculated at 32°C for seven days. Malassezia species were identified according to their macroscopic and microscopic features and physiological characteristics (14).

The macroscopic features of the predominant colonies included their shape, size, color consistency, and the characteristics of medium around them.

Microscopic features of the yeast cells in culture were described after lactophenol staining and included the predominant morphology, size and budding base of the yeasts.

To assess the physiological properties of the yeasts catalase reaction was determined by using a drop of hydrogen peroxide (30%) onto a culture smear on a glass slide. The production of gas bubbles, indicative of release of oxygen, was considered a positive reaction.

Utilization of Tween compounds was done according to the test originally described by Guillot et al. (14) and later modified by Gupta et al. (15). Yeast suspension, obtained by inoculating 5 mL of sterile water with a loopful of actively growing yeasts, was inoculated on Sabouraud glucose agar. The inoculum was then spread evenly. Each plate was divided into four sections and 5 mL of Tween 20, 40, 60 and 80 were added into a hole made in center of each section and incubated for a week at 32°C. Utilization of Tweens was assessed by the degree of growth and/or reaction of the lipophytic yeasts around individual holes.

3. RESULTS

From PV lesions all samples gave positive cultures. The most commonly isolated species was M. globosa (65%), followed in frequency by M. sympodialis (15%), M. furfur (10%), M. obtusa (7.5%) and M. slooffiae (2.5%). M. restricta and M. pachydermatis were not recovered from our patients.
The most frequently isolated species from the lesions of SD were M. restricta (27.5%), M. globosa (17.5%) and M. slooffiae (15%). Both M. sympodialis and M. furfur were cultured in 12.5%. M. pachydermatis was isolated in one case (2.5%). The percentage of negative culture was 12.5%.

Malassezia yeasts were found in 92.5% samples taken from scalp skin of psoriatic patients. The most frequently isolated species was M. globosa found in 55% patients, followed by M. slooffiae (15%), M. furfur (7.5%), and M. sympodialis (2.5%). Two species, M. pachydermatis and M. obtusa, were not isolated.

The results of culture obtained from healthy subjects were positive for Malassezia yeasts in 72.5% cases. M. sympodialis was the dominant species (30%), although M. globosa and M. furfur were also frequently isolated, in 22.5% and 17.5%, respectively. M. restricta was identified in a single case (2.5%). M. obtusa, M. slooffiae and M. pachydermatis were not identified. Malassezia species isolated from patients with PV, SD and PS and from healthy subjects are demonstrated in Table 1.

4. DISCUSSION AND CONCLUSION

M. pachydermatis was first described in 1925, when Weidman observed yeast-like cells in scales from cutaneous lesions of Indian rhinoceros (Rhinoceros unicornis) (16). Since then the importance of M. pachydermatis has been documented in both veterinary and human medicine. The most commonly described human infection due to M. pachydermatis is catheter-related fungemia in premature infants receiving intravenous lipid alimentation (1, 11, 17).

The route by which M. pachydermatis was introduced into the intensive care nursery is not certain. The cutaneous commensal flora of the patient or health care workers, after being colonized from pet dogs, is usually the source of infecting organism (18). It has been shown that the skin of a dog can be colonized by more than one type of M. pachydermatis (19), but a single strain of M. pachydermatis, as determined by pulsed-field gel electrophoresis (PFGE), was isolated from patients, health care workers and from dogs owned by healthcare workers (18). According to a report of a positive vaginal culture, another possibility is that an infant became colonized during transit through the birth canal at delivery (20).

In humans, reports of skin infections have been rarely reported than systemic infection due to M. pachydermatis. Somerville reported that 16% of patients with chronic cutaneous diseases harbored M. pachydermatis on the skin (21). One report from Thailand identified that 12% of 200 healthy subjects carried M. pachydermatis at sites on the head and palm from whom samples were collected by a washing technique for fungal culture, with subsequent speciation of yeast by biochemical methods (22). These findings were not confirmed by other investigators who, in most cases, could not isolate M. pachydermatis, or isolated this species infrequently (23). Recently, Nakabayashi et al. isolated various Malassezia species from the skin of healthy individuals and from patients with PV, SD, and atopic dermatitis using a swab technique for fungal culture. They found that M. pachydermatis was present in only 4% of patients with SD and in 5% of patients with PV and normal subjects (24).

In our study, out of 160 skin samples from diseased and healthy skin, M. pachydermatis was isolated in only one case, from lesional scalp skin of a 26-year-old male veterinary student. We speculate that the fungus isolated from the patient could be from dogs because of the patient’s close contact with dogs. Namely, it is a widely documented that dogs, especially atopic dogs, which are natural hosts for M. pachydermatis, represent a risk factor for human carriage. In a study conducted by Morris et al., the positive rates of M. pachydermatis according to conventional culture method and DNA amplification by nested PCR were 6% and 92% in skin samples of 50 owners of healthy dogs and 38.7% and 93.3% in 50 owners of atopic dogs, respectively, indicating that the transfer of M. pachydermatis from the diseased canine skin to the human skin occurs commonly (25).

M. pachydermatis is not a member of the normal human flora and the presence of this species on human skin indicates an external source of infection.

REFERENCES
10. Chen TA, Hill PB. The biology of Malassezia organisms and their ability to induce immune responses and skin dis-

<table>
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<tr>
<th>Malassezia Species</th>
<th>PV n (%)</th>
<th>SD n (%)</th>
<th>PS n (%)</th>
<th>HS n (%)</th>
<th>TOTAL n (%)</th>
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<tbody>
<tr>
<td>M. globosa</td>
<td>26 (65)</td>
<td>7 (17.5)</td>
<td>22 (55)</td>
<td>9 (22.5)</td>
<td>64 (40)</td>
</tr>
<tr>
<td>M. sympodialis</td>
<td>6 (15)</td>
<td>5 (12.5)</td>
<td>1 (2.5)</td>
<td>12 (30)</td>
<td>24 (15)</td>
</tr>
<tr>
<td>M. furfur</td>
<td>4 (10)</td>
<td>12 (30)</td>
<td>7 (17.5)</td>
<td>19 (11.9)</td>
<td></td>
</tr>
<tr>
<td>M. restricta</td>
<td>0</td>
<td>11 (27.5)</td>
<td>4 (10)</td>
<td>1 (2.5)</td>
<td>16 (10)</td>
</tr>
<tr>
<td>M. slooffiae</td>
<td>1 (2.5)</td>
<td>6 (15)</td>
<td>7 (17.5)</td>
<td>0</td>
<td>14 (8.7)</td>
</tr>
<tr>
<td>M. obtusa</td>
<td>3 (7.5)</td>
<td>0</td>
<td>0</td>
<td>3 (1.9)</td>
<td></td>
</tr>
<tr>
<td>M. pachydermatis</td>
<td>0</td>
<td>1 (2.5)</td>
<td>0</td>
<td>1 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>5 (12.5)</td>
<td>3 (7.5)</td>
<td>11 (27.5)</td>
<td>19 (11.9)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>160 (100)</td>
</tr>
</tbody>
</table>

Table 1. Malassezia species isolated from patients with PV, SD and PS and from healthy subjects. PV = pityriasis versicolor, SD = seborrhoic dermatitis, PS = psoriasis, HS = healthy subjects.
Identification of Malassezia Pachydermatis from Healthy and Diseased Human Skin

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