Short Communication

Two Possible Cases of *Trichosporon* Infections in Bone-Marrow-Transplanted Children: the First Case of *T. japonicum* Isolated from Clinical Specimens

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SUMMARY: *Trichosporon* spp. are emerging as opportunistic agents that cause systemic diseases in immuno-compromised hosts. Trichosporonosis carries a poor prognosis in neutropenic patients. *Trichosporon japonicum* was isolated from the air and named by Sugita et al. Here we present the first case of *T. japonicum* isolated from a clinical specimen. Two cases of acute myeloid leukemia who had *Trichosporon* isolates are discussed because of their rarity and growing importance. *T. asahii* was isolated from the throat, feces and urine of the first patient. *T. japonicum* was isolated from the sputum of the second patient. Both cases produced high MICs to itraconazole, and low MICs to fluconazole and voriconazole. In virulance factor investigations there was (++) biofilm formation in *T. japonicum* but not in *T. asahii*. Conventional mycological studies were not adequate for the identification of the isolate at the species level. In our second case as in the first one, the isolate was identified as *T. asahii* with 99.9% accuracy by API 20C AUX. Although two *T. asahii* isolates from the same patient yielded identical typing profiles by arbitrary primed-PCR, the isolates of the two different patients showed different arbitrary primed-PCR typing profiles. However, the genetic identification of the other patient's strain gave the result of *T. japonicum*.

Case 1. A 22-month-old boy with acute myeloid leukemia (AML) was admitted for autologous bone marrow transplantation (BMT) in July 2000. The patient was given fluconazole (FLU) prophylaxis according to routine protocol. His absolute neutrophil count (ANC) was below 500/mm³ until day +53 and he had several febrile neutropenic attacks. Due to fever-of-unknown-origin treatment protocol in neutropenic patients, he was given multiple antibiotics and liposomal amphotericin B (AMB). His hemocultures were all sterile. His physical examination revealed numerous squamous and hyperkeratotic lesions. He had icterus and diarrhea. Yeast was isolated in his throat, urine and stool cultures, and identified as *Trichosporon asahii*. Urine cultures yielded yeast colonies (900 cfu/ml) that lasted 4 days. This time he developed hematuria and proteinuria. Cultures of the catheter exit site and sputum were negative. One of four blood cultures at different times yielded methicillin-resistant coagulase-negative *Staphylococci* (MRCNS) and the others were negative. Itraconazole (ITRA) was included in the therapy. Fortunately, the patient's ANC increased to >1,000/mm³ at the 2nd day after identification of *T. asahii*, and his fever resolved after combination therapy with liposomal AMB (4 mg/kg) + ITRA (100 mg/d) + FLU (400 mg/d). But he had high C-reactive protein (CRP) values, ranging between 38.3 to 228.2 mg/l, during his follow-up. The combination therapy was continued and he was discharged from the BMT unit at day +103. He continued to recieve liposomal AMB and ITRA for 6 more months in the outpatient clinic and he recovered. At +11 month he experienced a relapse. He succumbed to death in deep neutropenia and relapse 12 months after BMT without *T. asahii* isolation.

Case 2. An 8-year-old girl with AML was admitted for allogenic BMT in January, 2000. She was discharged from the BMT unit without any problem. She developed central nervous system relapse followed by bone marrow relapse and was hospitalized. Idarubicin (IDA) treatment regimen was applied (day +210 post BMT). When she was in deep neutropenia (ANC, 200/mm³), she developed a high fever with CRP of 200 mg/l. She was given multiple antibiotics and liposomal AMB (5 mg/kg). No improvement was observed. On her physical examination, she had secretory ralles and was under respiratory distress. Liposomal AMB treatment (5 mg/kg) was started; later, the dose was increased. Sputum cultures taken many times during 11 days yielded as follows: *Aspergillus fumigatus*/yeast (unidentified) + *Pseudomonas* spp./yeast/yeast + *A. fumigatus*/*Trichosporon japonicum* (identified after death) + MRCNS (122 days after BMT)/ *Candida famata* + *Candida tropicalis* + MRCNS.

Physical examination of the pulmonary system revealed harsh bronchial sounds and crepitant rales. She had a fever that lasted for a week. ITRA (100 mg/d) was added to the treatment. The stool culture yielded *C. tropicalis*, and the throat culture yielded *C. famata*. Later, the general condition of the patient deteriorated. On physical exam, she had secretory rales and was experiencing respiratory distress. Then, wheezy breathing started and continous respiratory support with oxygen was applied. After she intubated, she was sent to the intensive care unit. Two days later, she died, 8 months after BMT.

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The fungi were identified based on morphology and using API 20C AUX (bioMérieux, Marcy l'Etoile, France). In both cases excellent *T. asahii* identification profiles were obtained.

The intergenic spacer region (IGS) of the rRNA gene was sequenced directly from the polymerase chain reaction (PCR) products with the primer pairs 26SF (ATCCTTTGCAGAC GACTTGA) and 5SR (AGCTTGACTTCGCAGATCGG). The PCR products were sequenced with an ABI 310 DNA sequence and the BigDye Terminator Cycle Sequencing Ready kit (Perkin-Elmer Applied Biosystems, Foster City, Calif., USA) according to the manufacturer's instructions. The sequence data were analyzed with the National Center for Biotechnology Information (Bethesda, Md., USA) BLAST system (http://www.ncbi.nlm.nih.gov/BLAST/). Ribosomal DNA sequence analysis of throat and stool isolates of Case 1 showed identical sequences. Their sequences matched the IGS of *T. asahii* (GenBank accession no. AB066396). The IGS sequences of Case 2 sputum isolate corresponded to that of *T. japonicum* (GenBank accession no. AB066426).

The antifungal susceptibilities were determined with the E test system (AB Biodisk, Solna, Sweden). According to the results of reference laboratories, MIC breakpoints for voriconazole (VOR) are >4 μg/ml for resistance and <1 μg/ml for susceptibility (1). For other antifungals tested, Clinical and Laboratory Standards Institute (CLSI) reference microdilution MICs were used for building susceptibility database for *Trichosporon* isolates. *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 served as quality control organisms for all tests (2,3). For Case 1, the MIC values for FLU, AMB, ITRA and VOR were 2, >32, 0.75 and 0.047 μg/ml, respectively; for Case 2, MIC values were 0.032, 1.0, 0.75 and 0.032 μg/ml, respectively. Both cases produced high MICs to ITRA; the *T. ashaii* isolate had a higher MIC of AMB than *T. japonicum*. Both *Trichosporon* isolates produced low MICs to FLU and VOR.

The phospholipase test was performed on egg yolk containing agar medium (pH 4.3). The test strain was inoculated onto medium by means of a calibrated loop (0.01 ml) and incubated at 37°C for 4 days. At the end of this period, the precipitation zone formed around the colony was measured and the strength of the reaction was detected by calculating the ratio between colony diameter and the diameter of the precipitation zone including the colony size. A value of 1 showed a negative reaction while values smaller than this indicated +1, +2, +3 or +4, according to the millimetric measurements. *Candida albicans* sc 5314 was used as the positive control (4,5).

For secretory acid proteinase asssay, agar medium with 1% bovine serum albumin (pH 5.0) was used. From each test strain grown in yeast extract-peptone-dextrose broth with a turbidity of 0.5 McFarland, a 10-μl sample was inoculated onto a paper disk placed on the agar medium and incubated at 30°C for 6 days. An opaque precipitation was formed around the disk and was enhanced by ongoing growth. On the 3rd or 4th days the lysis zone around the disk (1 mm, +; 3-5 mm, ++; no lysis, −) was measured to determine the proteolytic activity of the strain. The CBS 2730 *C. albicans* strain was used as a positive control (4,6).

The esterase test was performed on Tween 80-containing agar medium (pH 6.8). Each strain inoculated onto the medium in a ring form was examined for the formation of opaque precipitates of opaque crystal resulting from the hydrolysis of Tween 80 and binding of fatty acids with calcium (7).

For slime test, Sabouraud broth with an 8% final concen-tration of glucose was inoculated with the test strain and incubated at 35°C for 48 h. After washing and staining with 1% safranin the intensity of the film layer formed inside the plastic tubes was evaluated as negative, weakly positive (+), moderately positive (++) or strongly positive (+++) activity. ATCC 35984 *Staphylococcus aureus* strain was used as positive control (8-10).

Isolate from Case 1 (stool): phospholipase (−), acid proteinase (−), esterase (+), biofilm (−). Isolate from Case 2 (sputum): phospholipase (−), acid proteinase (−), esterase (+), biofilm (++).

Molecular typing of the isolates was performed by arbitrary primed (AP)-PCR with M13 primer. For AP-PCR analysis, genomic DNA was extracted using a QIAamp DNA mini kit (Qiagen, Valencia, Calif., USA). The primer used in the PCR reaction was the core sequence of phage M13 (5′-GAGG GTGGCGGTTCT-3′). PCR amplification was performed in 50 μl master mix containing approximately 100 ng of DNA as template, 1× amplification buffer, 0.4 mM of dNTP mix, 4 mM MgCl$_2$ and 2.5 U *Taq* DNA polymerase (11). The reaction mixture was amplified with a Thermal Cycler (Corbett Research Palm-Cycler; Corbett Life Science, Sydney, Australia) according to the following conditions: 2 cycles, each consisting of 5 min at 94°C, 5 min at 40°C, and 5 min at 72°C; then 40 cycles, each consisting of 1 min at 94°C, 1 min at 40°C, and 2 min at 72ºC. Amplification products were electrophoresed by 2% agarose gel; after staining they were visualised and photographed under UV illumination.

AP-PCR typing showed that the *T. asahii* isolates from the same patient were identical (lanes 1 and 2, Figure 1); however, the isolates from the two patients were different (lanes 1 and 2 versus lane 3, Figure 1), clonally unrelated (Figure 1).

A yeast strain isolated from the air in Japan was found to represent a new species, and was named *T. japonicum* (1998) (12). Summer-type hypersensitivity pneumonitis (SHP) is a type 3 or 4 allergy developed by repeated inhalation of arthroconidia of *Trichosporon* spp. One of these *Trichosporon* spp. was *T. japonicum* (13,14). We have isolated *T. japonicum* from the sputum of a patient, as it is isolated from the air of SHP patients' houses. Further analysis of the yeast isolate could be performed after the patients had died. In order to investigate retrospectively the epidemiological relationship of *T. asahii* strains in two different patients at the same dates, we did a molecular typing study on these strains, which had been stored in a freezer since 2000. The yeast isolates were sent to Japan for the genetical confirmation of identification. The molecular typing results revealed that these three *Trichosporon* strains were two different species but with only the genetic study the identification was made as *T. japonicum* in 2006. Our case is the first isolation of *T. japonicum* from a clinical specimen (2000). In the first case excellent *T. asahii* identification profiles were obtained by API 20C AUX, as in several other studies. In our second case as in the first case, the isolate was identified as *T. asahii* with 99.9% accuracy by API 20C AUX; however, the genetic identification of this strain gave a different result, *T. japonicum*.

At first studies *Trichosporon asteroides*, which was the cause of the described blood-stream infections, could not be correctly identified using ID32 C strips. The recent studies of fungal 26S rDNA and ITS region sequence data facilitated the earlier and more reliable identification than phenotypic methods. Unfortunately, these sophisticated molecular methods are performed only in reference centers. Some identifica-
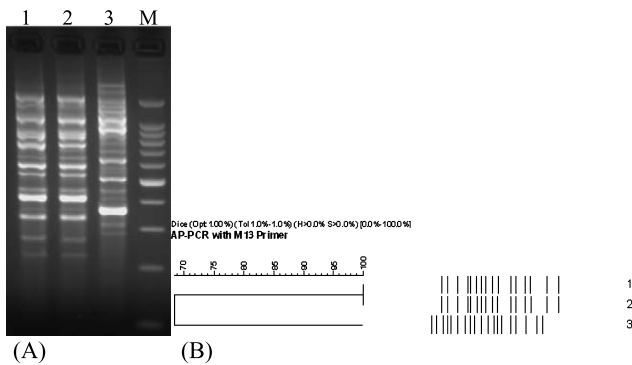
Fig. 1. (A) Agarose gel electrophoresis of the amplified products in AP-PCR with M13 primers. Lane1, Case 1 throat; lane 2, Case 1 stool; lane 3, Case 2 sputum isolates. Lane M is 100-bp DNA ladder. (B) A dendrogram of the DNA fingerprinting patterns of two *T. asahii* isolates (no. 1 and 2) and one *T. japonicum* isolate (no. 3).

tion system such as ID32 C and API *Candida* kits have been commonly used to identify pathogenic yeasts in clinical microbiology laboratories. Moreover, *T. asteroides* has not been included in the ID32 C system or other systems. Therefore, it seems impossible to identify clinical isolates of *T. asteroides* to the species level by their biochemical characteristics used in these systems (15). *T. japonicum* is also not included in the identification list of commercial kits. For this reason, in order to submit data for epidemiological studies, further genetic studies should be done for isolated *Trichosporon* spp. This should also include the reevaluation of past isolated *Trichosporon* spp. If we continue, we think that it will be possible to find more *T. japonicum* isolates.

Careful examination is required in diagnosing *Trichosporon* infection in immunocompromised hosts, because the infection can be invasive or unusual in appearance (16). Our first case had papulopustular and erythematous cutaneous lesions but first were evaluated as exhibiting a drug reaction. Knowledge of clinical manifestation of cutaneous infections with *Trichosporon* spp. should lead to earlier diagnosis and institution of appropriate treatment.

The two cases reported here were profoundly immunosuppressed and neutropenic at the time of isolation. Response to antifungal agents is poor, mortality is high, and immunological recovery is the most important factor for a favorable outcome in patients with trichosporonosis (17). We believe that in the first patient the immune reconstitution was the major determinant of possible trichosporonosis. Few cases with normal neutrophil counts who recovered from infection without antifungal therapy have been reported in the literature (18). Despite the poor clinical condition of the second patient, the exact contribution of *T. japonicum* to the patient's death is difficult to estimate. However, it would be very naive to assume that these pathogenic *Trichosporon* strains were not the causative pathogens in these patients.

In the two patients combination therapy was used because of the severtiy of the neutropenia and high CRP, which was thought to be a good indicator of infection severity in our patients. *Trichosporon* spp. may colonize and proliferate in different parts of the human body such as the oral cavity, gastrointestinal system, urinary tract or skin. Therefore, the presence of *Trichosporon* spp. in the sputum, feces and urine specimens does not indicate true infection (15). But in immunocompromised patients it may be life-threatening with a poor prognosis. Early effective treatment may be life-

saving. Diagnosis relies on clinical suspicion with microbiological confirmation (17).

In our cases although we could not isolate the strains from the blood culture, this may have been due to the difficulty of isolation. These findings do not directly correlate with the *Trichosporon* spp. infections in our cases. In conclusion, difficulties in the identification of *Trichosporon* spp. cause the delay of a more radical treatment and increase the mortality rate. Indeed, the mortality rate in these patients is estimated to be approximately 78% despite AMB therapy (19). In order to prevent such undesirable results, it would be more proper to insist on taking samples from suspected febrile neutropenic patients. However, clinical suspicion is essential for earlier usage of antifungal therapy to improve the outcome. Clinicians should be aware that *Trichosporon* fungemia may develop in patients under antifungal therapy.

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