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Recovery of lipophilic *Malassezia* species from two infants with otitis media in Monterrey, Nuevo León, Mexico

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Malassezia **species are yeasts that can be present on healthy human skin but also associated with external otitis and other skin diseases. The aim of this work was the identification of *Malassezia* -lipophilic- species recovered from infants with otitis media. Microbiological culture, polymerase chain reaction (PCR) and sequencing were performed for *Malassezia* detection in samples obtained from two cases of infants with otitis media. We identified two strains of lipid-dependent *Malassezia* species: *M. furfur* and *M. restricta*. This is the first report regarding the presence of *Malassezia* species in infants with otitis media in Monterrey, México.**

Key words: *Malassezia, Malassezia furfur*, *Malassezia restricta*, otitis media, infants, LSU rDNA D1/D2, yeasts.

INTRODUCTION

Malassezia species are lipophilic and /or lipid-dependent yeasts characterized by a thick cell wall, and are considered as normal components of the skin microbiota of both animals and humans (Crespo et al., 2000; Midgley, 1989; Batra et al., 2005).

Because of the difficult isolation and characterization of *Malassezia* spp. using traditional identification tests, molecular biology probes such as restriction fragment length polymorphism (RFLP) (Guillot et al., 2000; Gaitanis et al., 2002), pulsed-field gel electrophoresis (PFGE) (Gupta et al., 2004), random amplified polymorphic DNA (RAPD) (Castellá et al., 2006), and polymorphism amplified fragment length (AFLP) (Theelen et al., 2001) have been implemented. Actually, pyrosequencing techniques are

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used for a more precise and rapid identification of *Malassezia*, which as a consequence helps to implement more adequate and fast treatments (Kim et al., 2013). Other techniques, such as PCR-RFLP (Jagielski et al., 2014) and multiplex PCR methods (Vuran et al., 2014) have also been proposed and are useful to identify *Malassezia* species directly obtained from patients samples.

Based on molecular characteristics and lipid sources requirements, seven species of the genus *Malassezia* have been described, including *Malassezia furfur* (Robin) Baillon 1889, *M. pachydermatis* (Weidman) Dodge 1935, *M. sympodialis* Simmons & Gueho 1990, *M. globosa* Midgley, Guého and Guillot 1996, *M. obtusa*, *M. restricta* and *M. slooffiae*. *M. pachydermatis* is the only non-lipid-dependent species (Guého et al., 1996). More recently, the following seven new species, *M. dermatis*, *M. japonica*, *M. yamatoensis*, *M. nana*, *M. caprae*, *M. equina* and *M. cuniculi* were recently proposed (Sugita et al., 2002; Sugita et al., 2003; Sugita et al., 2004; Hirai et al., 2004; Cabañes et al., 2007 and 2011).

The presence of *Malassezia* associated with external otitis in carnivores such as dogs and cats is well documented (Eidi et al., 2011; Shokri et al., 2010; Hernández et al., 2012). However, there are few reports of this yeast in human cases of external otitis (Kaneko et al., 2010) and no reports of lipophilic *Malassezia* as causing agent of otitis media in infants and adults. Therefore, the aim of this work was to report the isolation and identification of *Malassezia* by PCR amplification and sequencing of the D1/D2 region of DNA encoding the LSU rRNA in two infants with a clinical diagnosis of otitis media.

MATERIALS AND METHODS

Isolation of *Malassezia* species

The samples were obtained from a 30-day old boy presented with a yellow-green secretion of the middle portion of right ear. The patient demonstrated excessive itching, tympanic hyperemia, and discharge in the ear canal. A diagnosis of otitis media was made based on otoscopic examination. The second sample was obtained from a 7-year old girl presented with bilateral secretion of the middle ear. A diagnosis of chronic otitis media was made. Swab samples of the discharge were taken. Both samples were seeded on potato dextrose agar and modified Dixon medium containing 0.5 g of chloramphenicol and cycloheximide.

Gram stain was performed in the grown colonies. The methodology for species identification was based in the ability to utilize individual Tween test proposed by Guého et al. (1996) and Tween diffusion test proposed by Guillot et al. (1996).

Identification of bacterial species

Bacterial isolation was done on blood culture media and S 110 media. For identification and morphology of microorganisms, Gram stains were performed. Characterization of recovered bacteria was performed by the automated system Vitek® (Laboratory bioMérieux). Briefly, a suspension of each cultured strains was prepared in a test tube with 0.45% of sterile saline solution. Tube suspension MacFarland No.1 ($3x10^8$ cells / ml) was adjusted. Gram negative identification (GNI) was extracted from the individual bag and marked with the strain number with Vitek®marker. The card was then packed in the module, sealed and placed in the reader / incubator module. Vitek® filling procedure was followed until the report was generated. Similarly, the process for Gram positive identification (GPI) card was conducted.

DNA extraction from pure cultures

Reference strains of *M. furfur* (CBS 1878^{NT}) and *M. restricta* (CBS 7878^{T}) along with clinical isolates were grown and maintained on modified Dixon's agar, containing 0.5 g of chloramphenicol and cycloheximide at 32°C for 8 days. DNA extraction was performed as previously described by Ferrer (2001). One hundred ng of genomic DNA was included in the following PCR reaction mixture to achieved final concentrations of 10 mM buffer 10X, 0.1 mM dNTP´s, 1.5 mM MgCl, 0.198 µM each primer (NL1: F- 5´ GCATATCAATAAGCGGAGGAAAAG-3´; NL4-R- 5´-GGTCCGTGTTTCAAGACGG -3´) (O'Donnell, 1993) and 1 U *Taq* DNA polymerase. Amplification was performed with 30 cycles (94°C for 45s, 51°C for 1 min and 72°C for 3 min) using a PTC-100 Pelter Thermol Cycler (MJ Research Inc, Massachussetts, USA). PCR products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide under a UV transiluminator (D&RI Ind. Ltd Transilluminator and Gel-Pro Imager). PCR products were sequenced in an ABI Prism 3130 (Applied Biosystems, Foster City CA, USA). The sequences were submitted to Genbank. The first patient was given oral Amoxicillin every 8 h for 7 days and 1% Bifonazole cream, once daily for 14 days. Patient 2 was given Clindamicyn twice daily and Clotrimazol 2 to 3 times per day for 14 days.

RESULTS

The microscopic observation revealed yeast showing monopolar budding. *M. furfur* was identified in the first patient (JY-1) while *M. restricta* was identified in the second patient (JY-2). In Table 1, a summary of the characteristics of the patient isolates compared with reference strains (*M. furfur* CBS 1878^{NT} and *M. restricta* CBS 7878^{T}) is presented. Bacterial species identified with the Vitek ® automated system (BioMérieux Laboratory, México, DF) were *Klebsiella pneumoniae* and *S. aureus* in patient 1 and *S. aureus* in patient 2. After D1/D2 region amplification and comparison to the reference strain *M. furfur* CBS 1878^{NT}, JY-1 and JY-2 yielded the expected PCR product of 600 bp characteristic of *Malassezia*. Sequence alignments confirmed that the isolate from patient 1 had from 99 to 100% homology to *M. furfur* at the nucleotide level (GenBank accession No. KC415103.1 and AY745725.1, respectively) and sequence from patient 2 had from 98 to 99% homology to *M. restricta* (GenBank accession No. AJ249950.1 and JN651957.1, respectively). The nucleotide sequence data reported from both cases are available in the DDBJ/EMBL/GenBank database under accession Nos. JF323946.1 and JX439915. Children healed after treatment.

DISCUSSION

Despite the fact that *Malassezia* is found as part of the

Table 1. Physiological characteristics of *Malassezia* species studied.

Parameter	Glucose/Peptone Agar 32°C (Guého et al., 1996)	Catalase (Guého et al., 1996;Guillot et al., 1996)	Growth with Tween (Guého et al., 1996)			Tween diffusion Test (Guého et al., 1996)				Esculin (Mayser et al., 1997)	Growth T° (Guého et al., 1996).	
			20	40-60[a]	80	20[b]	40[b]	60[b]	80[b]		37°C	40°C
M. furfur[1]	-	+	+	+	+	+	+	+	+	+/-	+	+
M. restricta[2]	-	-	-	-	-	-	-	-	-	-	+	-
JY-1*	-	+	+	+	+	+	+	+	+	+	+	+
JY-2[+]	-	-	-	-	-	-	-	-	-	-	+	-

[1]*M. furfur* CBS 1887NT; [2]*M. restricta* CBS 7877[T]. *(Genbank accssesion No. JF323946); [+](Genbank accssesion No. JX439915). [a]At least one source of Tween should show growth; [b]Grows throughout the agar surface, but may be slightly inhibited growth around the well.

normal microbiota, infection with the organism is rarely reported. Consistent with our findings, it is important to highlight that the low occurrence of *Malassezia* in otitis media cases may not be due to absence of the organism but rather to the lack of suitable culture media for isolation of the micro-organism in clinical labs. In general, children cases referred to pediatric hospitals are linked to bacterial infections (Olajide et al., 2012; Turner et al., 2002; Parra et al., 2011). In this study, we performed an exhaustive literature review of otitis reports and found few describing the presence of *Malassezia* in external otitis cases in humans. Shiota et al. (2009) reported the presence of this yeast in 5 of 63 patients with otitis (Shiota et al., 2009). The presence of *M. slooffiae* and *M. restricta* in the external auditory canal of clinically healthy people (Kaneko et al., 2010) while presence of *M. sympodialis* in a diabetic patient with external otitis has also been reported (Chai et al., 2000). Sugita et al. (2013) reported the presence of *M. globosa* and *M. restricta* in lesions of the external auditory canal and sole of the foot (Sugita et al., 2013, Zhang et al., 2012). It is known that otitis is primarily caused by bacterial action, however, the presence of *Malassezia* species in infants with otitis media is yet to be reported. While most species of *Malassezia* colonize lipid-rich areas of human skin, these yeasts can be found on any area of the body. Usually *Malassezia* species constitute 53-80% of total skin fungal population (Gao et al., 2010). The presence of yeast with the bacteria found in the present study could indicate the possibility that *Malassezia* spp. may be involved in otitis media in humans. However, a larger epidemiological study is warranted to determine the nature of this relationship (Makimura et al., 2000; Zhang et al., 2012). Findings of this study are clinically significant since these results contribute to further understand the possible effects of fungal presence on human skin.

This investigation is the first report of lipophilic *Malassezia* species in infants with otitis media.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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