In vitro growth versus inhibition of growth of *Malassezia pachydermatis* in the presence of the antibacterial drug gentamicin

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*Malassezia pachydermatis* is part of the normal cutaneous microbiota of most warm-blooded vertebrates and is associated with otitis externa and seborrhoeic dermatitis in dogs and cats. In this study, we evaluated the growth capacity of nine *M. pachydermatis* strains on Sabouraud medium in the presence of a high concentration of gentamicin. Strains of *M. pachydermatis* cultured on Sabouraud medium in the presence of 50 and 100 μg gentamicin ml⁻¹ displayed different growth patterns such as growth or lack of growth. We hypothesized that this difference in growth of *M. pachydermatis* strains was correlated with the different genotypes of the strains. Random amplification of polymorphic DNA (RAPD) was applied for genetic typing of *M. pachydermatis* isolates, derived from the external ears of house pet cats suffering from otitis externa. The *M. pachydermatis* strains were cultured on commercial or home-made Sabouraud medium supplemented or not with gentamicin. RAPD analysis demonstrated a genetic heterogeneity between each strain. In particular, five out of nine strains tested were able to form colonies in the presence of gentamicin. However, a correlation between *M. pachydermatis* genotype and growth capacity in the presence of gentamicin was not widely demonstrated.

INTRODUCTION

*M. pachydermatis* is a lipophilic, non-mycelial, and saprophytic yeast frequently isolated from the skin of normal, asymptomatic animals. It becomes an opportunistic pathogen when normal cutaneous conditions are altered, favouring its growth, thus causing skin infection (Bond, 2010; Nardoni et al., 2005; Shokri et al., 2010). Occasionally it is isolated from humans and has caused nosocomial infections in neonates, suggesting transference from pet animals (Chryssanthou et al., 2001). Recently, we demonstrated that *M. pachydermatis* is able to stimulate the innate immune response in infected human keratinocytes, indicating a possible role of this yeast as a human opportunistic pathogen (Buommino et al., 2013).

Yeasts of the genus *Malassezia* display many virulence factors used to colonize the host (Gaitanis et al., 2012). Among these, resistance to antimicrobial drugs may favour its viability and transmission, as seen in *Candida* spp. and *Aspergillus* spp. (Pfaller, 2012), although until now, resistance to antimicrobial drugs has not been a recognized virulence factor of *Malassezia* spp. Aspíroz et al. (2010) reported that *M. pachydermatis* is susceptible to all major antifungals and that high concentrations (25, 50 and 100 μg ml⁻¹) of gentamicin inhibit its growth.

Here, we tested the growth capacity of some strains of *M. pachydermatis* in the presence of gentamicin and hypothesized that this characteristic could be related to different genotypes.

METHODS

Samples and *M. pachydermatis* cultivation. Clinical cases of feline otitis externa were recruited from the clinic of the Department

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of Veterinary Medicine and Animal Production, University of Naples ‘Federico II’, Naples, Italy. Auricular swabs were recorded from cats of the same city but not in strict proximity, and only from those at the first infection. The auricular samples were obtained by the introduction of a sterile swab into both ear canals. The swabs were processed for cytological examination and culture, as described previously (Buommino et al., 2013). For gentamicin assays, the specimens were incubated in Sabouraud dextrose agar (Biolife Italiana) containing 50 μg chloramphenicol ml⁻¹ or Sabouraud dextrose agar containing 50 μg chloramphenicol ml⁻¹ plus 50 μg gentamicin ml⁻¹ (Biolife Italiana) at 31 °C for 7 days.

M. pachydermatis colonies were harvested, suspended in physiological saline solution at a density of 10⁶ cells ml⁻¹, corresponding approximately to an OD of 0.1, and 1 ml of this suspension was cultured on commercial or home-made Sabouraud agar, supplemented or not with 50 or 100 μg gentamicin ml⁻¹. In this experimental design, gentamicin-free control plates were always used for growing the isolates. There are no standard conditions of anti-gentamicin effects on Malassezia growth prescribed by Clinical and Laboratory Standards Institute guidelines. The cultures were examined daily for 7 days. Visible colonies were prepared by Gram staining for immersion microscopy examination. Yeast colonies were then stored at −80 °C for future identification of species by PCR. Colony morphology pictures were taken with a Canon Powershot G3 camera.

**DNA extraction.** The samples were collected after Sabouraud dextrose agar plating and placed in a 2 ml screw-cap tube containing 0.3 ml 1 % SDS (Sigma) and vortexed at high speed for 30 s. One half of the tubes were filled with 425–600 mm glass beads (Sigma) and the samples were vortexed at maximum speed for 30 s. The samples were allowed to cool for 30 s. This vortexing and cooling procedure was repeated a further four times to break open the cell walls. A total of 400 μl phenol:chloroform solution (1:1, v/v; Sigma) was then added, and the mixture was vortexed at high speed for 10 s, followed by centrifugation at 9,200 g for 2 min. A total of 350 μl of the aqueous phase was removed and placed in a new tube with 35 μl 3 M sodium acetate (pH 5.2) and 2 vols ice-cold 96 % ethanol (Sigma), vortexed and stored overnight at −20 °C. The samples were then precipitated by centrifugation at 10,000 r.p.m. for 20 min. The supernatants were aspirated and the pellets were washed by adding 1 ml 70 % ice-cold ethanol and centrifuged at 10,000 r.p.m. for 2 min. Finally, the pellets were air dried and resuspended in 50 μl sterile H₂O and stored at 4 °C.

**PCR analysis.** A nested amplification of DNA was performed. The first PCR was performed using a primer set for the internal transcribed spacer (ITS) located between ITS1 and ITS2, specific for fungal rRNA genes. The second two sets of PCR primers were designed to amplify either the ITS1 region (ITS1 forward and middle reverse primers) or the ITS2 region (middle forward and ITS2 reverse primers) in which differences in length among *Malassezia* spp. have been observed (Gemmer et al., 2002). One hundred nanograms of DNA was amplified in a reaction mixture containing 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 10 μM dNTPs, 10 μM ITS1 forward primer (5′-TCCTGGAGGGGAACTCGGG-3′), 10 μM ITS2 reverse primer (5′-TCCTCCCGCTTATGATG-3′) and 2.5 U Taq DNA polymerase (Roche Diagnostics) in a final volume of 25 μl. In the two nested PCRs, 1 μl first-round amplification product was added to 24 μl of a new reaction mixture containing 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 10 μM dNTPs, 10 μM ITS1 forward sense primer and middle reverse primer (5′-TCCTGGAGGGGAACTCGGG-3′), or 10 μM middle forward primer (5′-TCCTGGAGGGGAACTCGGG-3′) and ITS2 reverse primer, 2.5 U Taq DNA polymerase and deionized water. The amplification program included an initial step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final extension cycle at 72 °C for 7 min. The expected PCR products were 269 bp for the ITS1 forward/middle reverse primers and 531 bp for the middle forward/ITS2 reverse primers. The reaction was carried out in a DNA thermal cycler (Master Cycler Gradient; Eppendorf). The PCR product was sequenced by Primim (Milano, Italy) using a DNA Analyser 3730XL (Applied Biosystems). DNA alignment was performed by submitting the gene sequence obtained to GenBank.

**Random amplification of polymorphic DNA (RAPD).** The primers used in this study to amplify RAPD sequences were OPA2 (5′-TGGCGAGCTG-3′) and FM1 (5′-AGCGGGCTCCATGGCAGCC-3′). FM1 presented the best result. It was selected based on high-intensity bands, hypervariability and good definition of DNA fragments. The RAPD reaction mixture contained 100 ng genomic DNA, 10 μM FM1 primer, 10 μM dNTPs, 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl and 2.5 U Taq DNA polymerase in a final volume of 25 μl. The amplification program included an initial step at 94 °C for 5 min, followed by 40 cycles of 1 min at 94 °C, 2 min at 40 °C and 2 min at 72 °C, with a final extension cycle at 72 °C for 7 min. Reactions were performed using a DNA thermal cycler (Master Cycler Gradient). The PCR products were analysed by electrophoresis on a 1.8 % agarose gel in TBE and stained with ethidium bromide. The gels were photographed under UV light to record the results. The isolates were considered to be similar genetic types if they varied by up to two DNA bands of electrophoresis on polyacrylamide gel.

**RESULTS**

The isolates were identified phenotypically (by macroscopic and microscopic morphology) as *M. pachydermatis*. The yeasts showed short, oval to ellipsoidal cells, with a broad budding base and a distinct collarette, but globose cells were also present. Strains were identified as *M. pachydermatis* by their ability to grow on Sabouraud dextrose agar without olive oil supplementation after 96 h incubation at 37 °C.

To confirm the biochemical identification of the isolated yeast, nested PCR of the ITS1 and ITS2 regions of the ribosomal gene cluster was performed as reported previously (Buommino et al., 2013). Isolation of fungal DNA from *M. pachydermatis* samples resulted in amplicons of the expected sizes (Fig. 1), as reported in the literature (Gemmer et al., 2002). In addition, the molecular analysis by Primim confirmed that all the genome sequences derived from the yeast isolates were closely related to that of *M. pachydermatis* available in GenBank; sequence similarities were: strain 1, 99 %; strain 3, 96 %; strain 4, 99 %; strain 27, 96 %; strain 47, 99 %; strain 50, 99 %; strain 89, 99 %; strain 105, 99 %; and strain 106, 99 %.

To verify if all *M. pachydermatis* strains were able to grow in the presence of gentamicin, the yeasts were cultured on commercial Sabouraud medium or on home-made non-lipid medium containing Sabouraud agar supplemented with 100 μg gentamicin ml⁻¹. Five out of the nine strains tested were able to form colonies in the presence of 50 and 100 μg gentamicin ml⁻¹. In particular, *M. pachydermatis* strains 27 and 50 formed the highest number of colonies, whilst strains 47 and 89 showed moderate growth and sparse colonies after 7 days of growth (Table 1, Fig. 2).
Strain 3 formed only two colonies, whilst strains 1, 4, 105 and 106 were unable to grow in the presence of 50 and 100 µg gentamicin ml⁻¹.

To determine whether the growth capacity of some strains in the presence of gentamicin could be genetically assigned to a specific band pattern, the genetic variability within strains was analysed. The strains were subjected to RAPD analysis using two primers OPA2 and FM1. The latter displayed the overall highest grade of discrimination and was selected to study the DNA polymorphism-based variability among the isolates (data not shown). The reproducibility of the RAPD patterns was assessed by carrying out a number of experiments in triplicate. No significant differences in the triplicate samples were observed, indicating the robustness of the conditions used (data not shown). As illustrated in Fig. 3 the strains of *M. pachydermatis* presented different genotypes. Isolates with identical banding profiles were grouped into four different genotypes as follows: genotype 1 for strains 3, 4 and 27; genotype 2 for strains 1 and 50; genotype 3 for strains 47 and 89; and genotype 4 for strains 105 and 106 (Table 1). From these results, it emerged that only genotypes 3 and 4 were associated with a particular phenotype (moderate and no growth, respectively).

**DISCUSSION**

Gentamicin is an aminoglycoside antibiotic composed of a mixture of related gentamicin components and fractions and is used to treat many types of bacterial infection, particularly those caused by Gram-negative microorganisms (Moulds & Jeyasingham, 2010).

Aspíroz *et al.* (2010) reported that the use of plates with high concentrations of gentamicin can lead to misdiagnoses such as false-negative cultures of *M. pachydermatis* and also an erroneous classification of the yeast as a lipid-dependent species. Here, we reported that only four out of nine *M. pachydermatis* strains tested were unable to form colonies in the presence of high concentrations of gentamicin. The remaining strains were able to grow, displaying different behaviours such as the number of colonies.

Generally, therapy in cats and dogs with otitis externa includes a combination of medications with antibacterial (gentamicin sulfate), anti-inflammatory and antifungal actions, a classical triple-action treatment. It is well known that antibiotic resistance is the result of resistant bacteria that can spread between different ecosystems and be selected by the widespread use of antibiotics in animals and

### Table 1. Genotype/phenotype correlations of *M. pachydermatis* isolates

<table>
<thead>
<tr>
<th><em>M. pachydermatis</em> strain no.</th>
<th>RAPD genotype</th>
<th>Sabouraud/gentamicin phenotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1</td>
<td>No growth</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>Low growth</td>
</tr>
<tr>
<td>27</td>
<td>1</td>
<td>High growth</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>No growth</td>
</tr>
<tr>
<td>50</td>
<td>2</td>
<td>High growth</td>
</tr>
<tr>
<td>47</td>
<td>3</td>
<td>Moderate growth</td>
</tr>
<tr>
<td>89</td>
<td>3</td>
<td>Moderate growth</td>
</tr>
<tr>
<td>105</td>
<td>4</td>
<td>No growth</td>
</tr>
<tr>
<td>106</td>
<td>4</td>
<td>No growth</td>
</tr>
</tbody>
</table>

*No growth, 0 colonies; low growth, ≤10 colonies; moderate growth, between 10 and 50 colonies; high growth, ≥50 colonies.*
in humans. Environmental exposure to gentamicin might, thus, contribute to the selection of gentamicin-resistant bacteria or fungi. In particular, animals with recurrent yeast infections, receiving gentamicin, might favour the selection of resistant strains. Antibiotic use has already been suggested as a cause of *Malassezia* spp. overgrowth and dermatitis (Patterson & Frank, 2002).

Furthermore, fungi can use codon reassignment to explore the available ecological landscape, thus discarding the theory of a universal genetic code. A study by Bezerra et al. (2013) reported that CUG ambiguity in *Candida albicans* leads to more genetic and phenotypic diversity, which allows the fungus to adapt to the environment. This could lead not only to changes in the pathogenesis of the yeast but also to the acquisition of drug resistance. Hofer (2013) discussed the genetic ambiguity and the genetic changes occurring on the basis of *C. albicans* cell-wall alterations, hypothesizing that CUG ambiguity affects *C. albicans* pathogenesis, because cell-wall changes might affect the recognition of fungal pathogen-associated molecular patterns by innate immune cells.

In this study, RAPD analysis showed genetic diversity between *M. pachydermatis* isolates, but, so far, we did not find a clear correlation among RAPD profiles, colony phenotypes and resistance to gentamicin. The only exception was for strains grouped in genotypes 3 and 4: strains 47 and 89 (genotype 3) formed colonies, whilst strains 105 and 106 (genotype 4) did not. However, it will be necessary to increase the number of strains tested to better define the relationship between genotype and phenotype.

The implications of these results raise the question of whether micro-organisms with different genotypes adapt to a new habitat and colonize using all possible strategies. Consequently, the elevated instability of the fungal genetic code leading to the generation of a new phenotype might produce new resistant strains, thus invalidating the therapeutic protocols. Our results clearly demonstrated that five out of nine *M. pachydermatis* strains were able to grow in the presence of gentamicin. If a correlation between genotype and phenotype could be confirmed, an accurate analysis of the strain genotype might lead to new therapeutic protocols by improving the management of fungal infections.

In conclusion, another question to be discussed: whether the different growth capacity of *M. pachydermatis* isolates in the presence of gentamicin is intrinsic, acquired or clinical. Further studies are needed to answer this question.

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