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Isolation and characterization of *Malassezia* spp. in healthy swine of different breeds

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ABSTRACT

Malassezia spp. genus is represented by several lipophilic yeasts, normally present on the skin of many warm-blooded vertebrates, including man. Swine are one of the less investigated animal species. The aim of the present work was to study the occurrence of *Malassezia* spp. in the external ear canal of 408 healthy swine of different breeds, under different breeding conditions. For this purpose N. 185 free-ranging wild boars, N. 107 large size pigs and 116 Cinta Senese breed were selected. Animals were of both genders, with age ranging from 8 months to 4 years. The subjects were culturally and molecularly checked for *Malassezia* spp. Ninety-two out of 408 animals scored positive for *Malassezia* yeasts (22.5%). *Malassezia pachydermatis*, *Malassezia sympodialis* and *Malassezia furfur* were recognized. *M. pachydermatis* was the sole species isolated from wild boars (12.9%), Cinta Senese (20.7%) and juvenile large size pigs (13.6%); 88% of large size breeds adult subjects scored positive for *M. sympodialis* (63.6%) and *M. furfur* (22.7%), respectively. The study focus on scarcely investigated epidemiological aspects of *Malassezia* spp. in this animal species.

1. Introduction

Malassezia genus encounters lipophilic yeasts, normally present on the skin of many warm-blooded vertebrates. Based on morphological, ultrastructural, physiological, and genetic features, seven species were assigned to the genus in the 90s (Guého et al., 1996; Guillot and Guého, 1995), *Malassezia pachydermatis*, the only non-lipid-dependent species, and the six lipid-dependent species *Malassezia furfur*, *Malassezia globosa*, *Malassezia obtusa*, *Malassezia restricta*, *Malassezia slooffiae* and *Malassezia sympodialis*. Further lipid-dependent species, namely *Malassezia dermatis* (Sugita et al., 2002), *Malassezia japonica* (Sugita et al., 2003), *Malassezia nana* (Hirai et al., 2004), *Malassezia yamatoensis* (Sugita et al., 2004), *Malassezia equina* and *Malassezia caprae* (Cabañes et al., 2007) were then described. These fungi have a role in otitis externa and seborrhoeic dermatitis of animals, mainly carnivores, as well as in *pityriasis versicolor*, seborrhoeic dermatitis, atopic dermatitis and folliculitis of man. In humans, *M. pachydermatis* was the responsible agent of infection both in immunocompetent (Ming Fan et al., 2006), and in immunocompromised subjects (Midgley, 2000); lipid-dependent species alone or variously associated among them or with *M. pachydermatis* are described in domestic animals (Crespo et al., 2002a,b; Nardoni et al., 2004).

Swine seem to be one of the less investigated animal species. Some reports prior to the description of the new species demonstrated the occurrence of *Malassezia* spp. on swine skin (Gustafson, 1959, 1960; Dufait, 1985; Kuttin and Glas, 1985; Guillot et al., 1994) with a marked predominance of lipid-dependent yeasts. Garau et al. (2005) identified *M. sympodialis* and *M. slooffiae* from 73% of ear canals in dermatologically healthy animals. To the best of our knowledge, there are no reports about the

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presence of *Malassezia* spp., related to breed and management in this animal species. Aim of the present work was to investigate the occurrence of *Malassezia* spp. in the external ear canal of different swine breeds, under different breeding conditions.

2. Materials and methods

External ear canals from 408 healthy swine were culturally and molecularly checked for *Malassezia* spp. The animals were of both genders, with age ranging from 8 months to 4 years. Animals aged more than 1 year were classified as adults. On the basis of breed, the animals were divided into 3 groups: free-ranging wild boars (N. 185), large size swine, such as Landrace, Large White and their crosses (N. 107) and Cinta Senese breed (N. 116). The first group consisted of 68 juvenile and 117 adult wild boars, the second comprehended 64 juvenile and 43 adults, while the last one 42 juvenile and 74 adult subjects. Large size pigs were intensively raised in 3 farms (Nos. 1, 2, 3), as well as Cinta Senese subjects were managed with extensive/outdoor production system in 3 different farms (Nos. 4, 5, 6).

Samples were collected by means of sterile cotton tip swabs immediately after killing during wild board hunting season, and at the swine farm in the others.

After collection, the specimens were promptly seeded onto sabouraud dextrose agar added with 0.5% of chloramphenicol and cycloheximide (Mycobiotic Agar®, DID, Milano, Italy) and mDixon Agar (3.6% malt extract, 0.6% peptone, 2% desiccated ox-bile, 1% Tween 40, 0.2% glycerol, 0.2% oleic acid, 1.2% agar, 0.5% chloramphenicol and 0.5% cycloheximide). The plates were incubated at 30 °C for 7 days and processed as previously reported (Nardoni et al., 2004). Briefly, lipid-dependent species were morphologically and biochemically identified, as described by Guého et al. (1996). The presence of *M. furfur*, *M. sympodialis* and *M. slooffiae* was proven by the Tween diffusion test, on the basis of their ability to assimilate various polyoxyethylene sorbitan esters. Bond and Anthony (1995) demonstrated the possible lipid dependence of some isolates of *M. pachydermatis*. Also, the presence of some lipids in the primary culture from the specimen can allow the growth of *M. furfur* in lipid-free medium. For these reasons, subsequent transfers were performed to clearly distinguish these two species. The identification of *M. furfur* was confirmed by using the Cremophor EL (Sigma, St. Louis, MO, USA) assimilation test, as reported by Raabe et al. (1998). The splitting of esculin was also performed as additional key to identify both *M. furfur* and *M. sympodialis* (Mayser et al., 1997). The lack of catalase activity, which is a specific feature of *M. restricta*, proved the presence of this species.

Morphological and biochemical identification was confirmed by means of a PCR-based technique using restriction enzyme digestion, specific for the discrimination of 11 *Malassezia* species, as described by Mirhendi et al. (2005). In order to achieve pure cultures, for each positive sample five colonies were subcultured onto mDixon Agar and stored at −20 °C until analysis. The cell walls were mechanically disrupted by freeze–thawing and genomic DNA was extracted and purified according to the DNeasy™ protocol for animal tissue (QIAGEN Inc., Valencia, CA, USA). The primers selected for this protocol amplify the target part of 26S rDNA, providing a single PCR product of an expected size of 580 bp. The PCR products were subjected to REA using CfoI and BstF51, separately, according to the manufacturer's instructions (Fermentas International Inc., Burlington, Ont., Canada). Digested fragments were analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide.

Yeast strains with morphological, physiological and molecular characters consistent with *M. sympodialis* were discriminated from the newly described species *M. caprae*, and *M. equina*, amplifying by PCR the variable D1 and D2 regions of the 26S rRNA gene, and the ITS and 5.8S rRNA gene. Isolation of DNA, PCR amplification process and DNA sequencing of rRNA genes of the isolates were performed as described by Cabañes et al. (2007). Statistic analysis to evaluate significant differences among breeds and age of pigs was performed by chi square test ($P < 0.01$).

3. Results

Ninety-two out of 408 animals scored positive for *Malassezia* spp. (22.5%). Three different species were recognized, namely *M. pachydermatis*, *M. sympodialis* and *M. furfur*. All *M. pachydermatis* isolates grew without lipid supplementation. Some of the lipid-dependent isolates grew scarcely, making difficult conventional identification. However most of the lipid-dependent species showed a strong catalase reaction, scored positive for assimilation of Cremophor EL (*M. furfur* isolates) and the splitting of esculin (*M. sympodialis* isolates) and were inhibited from Tween 20 only (*M. sympodialis*). PCR-RFLP results agreed with cultural findings, and resulted discriminating for isolates with a reduced growing rate, generating fragments identificative for *M. pachydermatis*, *M. sympodialis* and *M. furfur*, respectively. The sequences of the isolates identified as *M. sympodialis* on a preliminary count, resulted completely identical to *M. sympodialis* CBS 7222.

M. pachydermatis was the sole species isolated from both wild boars and Cinta Senese breed, being cultured from 24 animals for each group (12.9% and 20.7%, respectively). The prevalence of recovery for *Malassezia* spp. in large size animals was 44.1%; *M. sympodialis* was cultured from 28 subjects (63.6%), *M. furfur* from 10 (22.7%) and *M. pachydermatis* from 6 (13.6%). All yeasts species were obtained as a pure culture. Table 1 shows the prevalences of *Malassezia* spp. recovery from different swine breeds, based on farm and age of animals.

Animals harboring *Malassezia* species were present in two out of three intensive breeding farms, while all farms in which Cinta Senese were raised yielded yeasts, with different prevalences. Lipid-dependent species among large size breeds were limited to one farm only, where 88.3% of adults scored positive. Statistical analysis showed significant differences among overall prevalence values obtained from wild boars and Cinta Senese on one hand, and large size swine, on the other hand ($P > 0.01$).

Among juvenile wild boars, *M. pachydermatis* was isolated from 3 subjects only (4.4%), while this same species was recovered from 23/117 adults (19.6%). Thirty-eight out