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1 Comparison of two inoculation methods for *Microsporum canis* culture 2 using the toothbrush sampling technique

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11 12 13 14 15 **Background**

16
17 The toothbrush method is an effective method for obtaining material for fungal cultures.
18 However, the correct technique for inoculation onto the agar surface does not appear to
19 have been formally studied

20 21 **Hypothesis/objectives**

22
23 This study compared two inoculation techniques; the first involved pressing the toothbrush
24 onto the plate surface (procedure A), and the second involved pressing the toothbrush
25 onto the agar, as well as transferring hairs and scales entrapped in the bristles (procedure
26 B).

27 28 **Animals**

29
30 A total of 26 cattery-housed cats were sampled using the toothbrush technique. An
31 individually-packaged new toothbrush was longitudinally combed for 3 min over the hair
32 coat of each cat.

33 34 **Methods**

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36 The toothbrushes from each cat were then randomized to procedure A or B and the
37 investigator was blinded to inoculation technique. Cultures were performed on a medium
38 specific for dermatophytes. Results were compared considering the number of positive
39 plates along with other parameters such as the presence and abundance of colonies of
40 dermatophytes and contaminant moulds.

41 42 **Results**

43
44 A total of 21 cats were culture-positive for *Microsporum canis*. Procedure A allowed a
45 significantly higher number of positive plates (20/21) to be obtained compared with
46 procedure B (7/21). These results were mainly due to the higher plate invasion by
47 contaminant moulds, which was evident using procedure B.

48 49 **Conclusions and clinical importance**

50
51 This study provides evidence that fungal cultures should be performed by pressing
52 toothbrushes onto agar plates without including hair or scales.

53
54

55 **Introduction**

56

57 Dermatophytosis is a common fungal infection of cats, with *Microsporum canis* considered
58 to be the most important etiological agent.¹ This fungus is found worldwide and plays an
59 important zoonotic role. In some countries, *M. canis* tends to surpass anthropophilic
60 dermatophytes as a cause of human infections.¹ Dermatophytosis can present with a wide
61 variety of clinical signs; therefore, confirmation of infection relies on results from different
62 diagnostic tests. Fungal culture is normally considered the test of choice², and sampling
63 techniques for culture vary according to the situation.^{3,4} The “toothbrush method” is
64 recommended in cats with generalized lesions or subclinical infections.⁴ This represents a
65 variant of the method originally described by McKenzie *et al.*⁵, who employed hairbrushes
66 to detect scalp dermatophytosis in children. This method involves combing a human
67 toothbrush (considered mycologically sterile while in its packaging⁴) over the entire hair
68 coat in order to accumulate hair and keratin debris, followed by pressing onto the surface
69 of the culture plate.⁴ While this method is widely quoted^{1,2,6,7} and used,⁴ the correct
70 inoculation technique onto the agar surface has not been formally studied.⁴ Specifically,
71 since collected hairs tend to remain entrapped in the bristles despite repeated stabbing
72 onto the medium surface, it could be hypothesized that transferring hairs onto the plate
73 can increase the chance of obtaining positive cultures. Conversely, hairs are known to also
74 carry spores of contaminant fungi, and the growth of these fungi may negatively affect the
75 interpretation of culture results.⁴

76 This study was aimed at comparing two inoculation techniques of material collected by the
77 toothbrush method; the first involved purely pressing the toothbrush onto the agar surface,
78 and the second involved pressing the toothbrush onto the agar, as well as transferring
79 hairs and scales removed from the bristles to the plate.

80

81 **Materials and methods**

82

83 **Study population**

84

85 The study was conducted on 26 cats housed in a cattery with a history of recurrent
86 dermatophytosis. The cats lived in a rural area where they were allowed to freely roam.

87

88 **Sampling procedure**

89

90 Two new, individually-wrapped, human toothbrushes were used for each cat. Each
91 toothbrush was longitudinally combed for 3 min over the hair coat of each cat, starting from
92 the head, followed by the neck, dorsum, trunk, ventrum, limbs and tail. After specimen
93 collection, the toothbrushes were placed in new self-sealing plastic bags and transported
94 to the laboratory of (this information will be provided after the revision of the manuscript).

95

96 **Evaluation of hairs and scales**

97

98 Evaluation of the number of collected hairs and scales was carried out in the mycology
99 laboratory by a single investigator before plate inoculation. Examples reported in Figure 1
100 were used to assist scoring. The quantity of hairs and scales was evaluated as follows:

101

102 1. low (barely any visible material with the naked eye)

103 2. fair

104 3. abundant

105 4. very abundant (toothbrush completely covered by hairs entrapped in the bristles)

106

107 Fungal cultures

108

109 The toothbrushes from each cat were randomly allocated to inoculation procedure A or B
110 using a random choice generator (<http://jklp.org/html/choose.html>). For procedure A, the
111 toothbrush was pressed onto the surface of the agar (20 repetitions). Even in cases with
112 abundant or very abundant material, it was observed that most hairs and scales remained
113 entrapped in the bristles after pressing the toothbrush on the agar. With procedure B,
114 bristles were stabbed onto the agar surface (20 repetitions). Subsequently, all hairs and
115 scales entrapped in the bristles were removed by flame-sterilized hemostats and pressed
116 gently onto the agar surface.

117 Cultures were performed on Mycobios Selective Agar (Biolife, Milan, Italy) (formula per
118 litre: soy peptone 10 g; glucose 10 g; cycloheximide 0.4 g; chloramphenicol 0.05 g; agar
119 15 g). Plates were incubated at 25°C⁶ and examined daily for 2 weeks by a mycologist
120 blinded to the inoculation technique. Fungal colonies were identified to species level based
121 on their morphology and microscopic features.⁴

122

123 Comparison of the procedures

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125 Results obtained using the two procedures were compared considering the following
126 parameters:

127

- 128 • Number of plates with a positive result (growth of dermatophyte colonies).
- 129 • Number of plates with non-dermatophytic contaminant moulds (NDM).
- 130 • Number of colony-forming units (CFUs) of dermatophytes and NDM per plate.
- 131 • Degree of plate invasion by either dermatophytes or contaminating NDM, calculated
132 through an image processing and analysis program (imageJ, U.S. National Institutes of
133 Health, Bethesda, MD website, imagej.nih.gov/ij/), and expressed as the percentage of
134 plate surface (PPS) invaded by fungal colonies.
- 135 • Impact of the degree of plate invasion by contaminating NDM on the ease of visualizing and
136 sampling suspected dermatophyte colonies by microscopic examination. This parameter
137 was rated as follows (see Figure 2 for examples):

138

- 139 - PPS occupied by NDM < 25%. Visualization and sampling very easy
- 140 - PPS occupied by NDM 25 - 50%. Visualization and sampling easy
- 141 - PPS occupied by NDM 51-80%. Visualization and sampling difficult
- 142 - PPS occupied by NDM >80%. Visualization and sampling very difficult

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144 Statistical analysis

145

146 The prevalence of plates with dermatophyte colonies and NDM from the two procedures
147 was compared by the Chi square test, while the Wilcoxon rank-sum test with continuity
148 correction was used to compare the number of CFUs and the PPS. All of the analyses
149 were performed with R Core Team software (2014) (<http://www.R-project.org/>). A P-value
150 of < 0.05 was considered statistically significant.

151

152 Results

153

154 A total of 21 cats were culture-positive, with *M. canis* being the only dermatophyte isolated.
155 The quantity of hairs and scales collected on the two toothbrushes from each cat was
156 equivalent in all cases. Specifically, the quantity was rated as low in 4 cases (19%), fair in
157 2 cases (9.5%), abundant in 10 cases (47.6%) and very abundant in 5 cases (23.8%).

158 A summary of the culture results is provided in Table 1, while individual results can be
159 found in Table S1 (supplementary material). Procedure A allowed a significantly higher

160 number of positive plates (20/21; 95.2%) to be obtained compared with procedure B (7/21;
161 33.3%) ($\chi^2 = 17.53$, $p < 0.01$). There was no significant difference regarding the number of
162 plates with NDM. However, the number of NDM CFUs and the PPS invaded by NDM were
163 significantly higher in plates inoculated using procedure B. Conversely, for *M. canis* the
164 number of CFU and the PPS were significantly higher in plates inoculated using procedure
165 A (Figure S1). Differences were also noted regarding the ease of visualizing and sampling
166 *M. canis* colonies (e.g. 80% of plates were considered easy/very easy in procedure A
167 compared to 43% plates in procedure B, Table 1). However, a statistical comparison for
168 this parameter was not possible due to the low number of positive plates obtained in
169 procedure B.

170

171 **Discussion**

172

173 This study shows that the diagnostic value of fungal culture using the toothbrush technique
174 is heavily affected by the way the plate is inoculated. Specifically, transferring hairs and
175 scales from the toothbrush bristles to the agar (procedure B) only allowed isolation of *M.*
176 *canis* in 33% of cases, while significantly better results could be obtained when the
177 toothbrush was purely pressed onto the agar surface. These results indicate that cultures
178 can be positive even if most material (hairs and scales) remain on the bristles. This is likely
179 due to the fact that very small infected hair fragments and scales, and also free fungal
180 elements (arthroconidia), are transferred to the plate by pressing the toothbrush onto the
181 agar.

182 Plates inoculated with hairs and scales (procedure B) were frequently invaded by a high
183 quantity of NDM, so that the space in the plate became unavailable for the dermatophyte
184 colonies. For some samples, a nearly complete invasion of the plate by NDM was
185 observed (see Figure 2d and Table S1). The significantly higher invasion of the plate
186 surface by NDM appears to be the main reason for the delusory results obtained by
187 procedure B (only 33% positive plates vs. 95% obtained by procedure A). Inoculating hairs
188 on the medium surface is thus not only unnecessary, but even detrimental. The fact that
189 NDM colonies grew in the plates – in some cases very abundantly – despite the use of a
190 NDM growth inhibitor (cycloheximide) is not, however, surprising. The presence of NDM
191 colonies in cultures from cutaneous samples is a “normal” occurrence in the veterinary
192 laboratory^{2-4,8}, since the animal hair coat harbours a variegated fungal flora⁸, and
193 cycloheximide is not equally effective against all NDM species.⁹

194 Another advantage of procedure A is that the abundance of *M. canis* colonies, coupled
195 with the scarce NDM contamination, made it easy or very easy in most positive plates
196 (80%) to visualize and sample the colonies for microscopic confirmation. It should also be
197 noted that the number of *M. canis* colonies is a parameter that helps discriminating
198 between animals exposed to fomite contamination and cats with an active infection. It is
199 also useful to monitor the course of infection during treatment.³ Regarding procedure B, in
200 addition to the already mentioned overall poor performance, in more than half of the
201 positive plates (57%) the individuation and sampling of suspected colonies resulted difficult
202 or very difficult.

203 In conclusion, this study provides evidence that the correct technique to inoculate fungal
204 cultures when using the toothbrush technique consists of stabbing bristles onto the agar
205 without plating hairs and scales plucked from the bristles.

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207 **Supplementary material**

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209 Table S1. Individual results of cultures using procedure A and procedure B

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Table legend

Table 1. Results of cultures obtained using two different procedures of inoculation

Figure legends

Figure 1. Evaluation of the quantity of hairs and scales collected after brushing. Examples of (1) Low quantity. (2) Fair quantity. (3) Abundant quantity. (4) Very abundant quantity.

Figure 2. Examples of culture plates obtained in the study. Visualization and sampling of suspected *M. canis* colonies assessed as: a) very easy; b) easy; c) difficult; d) very difficult. Colonies marked with * = *M. canis*. Colonies marked with ° = non dermatophytic moulds (NDM)