Polish Journal of Microbiology
2011, Vol. 60, No 2, 145–148

ORGINAL PAPER

Strains Differentiation of *Microsporum canis* by RAPD Analysis Using $(GACA)_4$ and $(ACA)_5$ Primers

ANITA DOBROWOLSKA[1]*, JOANNA DĘBSKA[1], MAGDALENA KOZŁOWSKA[2] and PAWEŁ STĄCZEK[1]

[1]Department of Genetics of Microorganisms, University of Łódź
[2]Department of Dermatology and Venerology, Medical University of Łódź

Received 15 December 2010, revised 21 January 2011, accepted 2 February 2011

Abstract

Molecular analysis of dermatophytes (based on PCR fingerprinting) revealed high clonal differentiation between the genus and species. *Microsporum canis* (zoophilic dermatophyte, belonging to genus *Microsporum*), responsible for most cases of *tinea capitis* in children, *tinea corporis* in adults and dermatophytoses in cats, is very unique in comparison with other dermatophytes. Results of most molecular studies show that there is no clonal differentiation within *M. canis* as distinct from other species. The aim of this study was application of $(GACA)_4$ repetitive primer and $(ACA)_5$ primer for typing of *M. canis* strains isolated from human and animals in Central Poland. Fungal strains: 32 clinical isolates of *M. canis*, originated from patients from Central Poland; 11 strains isolated from infected cats (6) and dogs (7), reference strains of *M. canis* (CBS 113480), *T. rubrum* (CBS 120358), *T. mentagrophytes* (CBS 120357) and *E. floccosum* (CBS 970.95). The genomic DNAs of the strains were used as a template in RAPD reaction. No differentiation was observed for the analyzed *M. canis* strains using $(GACA)_4$ and $(ACA)_5$ typing.

Key words: *M. canis*, dermatophytes, strains differentiation, RAPD method

Introduction

Dermatophytes are a group of keratinophilic fungi causing infections called dermatophytoses. These microorganisms belong to the 3 genera of fungi, *Trichophyton*, *Microsporum* and *Epidermophyton*. *Microsporum canis*, zoophilic dermatophyte, is responsible for most cases of *tinea capitis* in children and *tinea corporis* in adults. The geographic spread of this infection is worldwide, because it is transmitted by cats (the principal reservoir of this fungus) or dogs. Human infection caused by *M. canis* may occur by direct contact with infected animals or with their hair. *M. canis* infections were also reported in humans who did not have a history of exposure to animals, which suggests that the infection may be spread indirectly from other humans colonized with the fungus or by contact with arthrospores that have contaminated object such as grooming equipment, furniture or environment (Weitzman and Summerbell, 1995).

Molecular PCR-based techniques provide a means of comparing isolates from patients and domestic animals to determine a source of contamination. Faggi *et al.* (2001) described the application of PCR for molecular identification and differentiation of dermatophyte species and strains using a simple repetitive oligonucleotide $(GACA)_4$ as a primer, which was previously successfully used by Meyer *et al.* (1993, 1997) to distinguish strains of *Cryptococcus neoformans* and *Candida* species. In case of clinical strains of *M. canis* isolated in Italy from humans, cats and dogs, the authors obtained only species-specific profiles, there was no intraspecies variation among them. The same results were obtained by Shehata *et al.* (2008) who also employed $(GACA)_4$ primer for molecular analysis of dermatophytes isolated from Egyptian patients. Our results using PCR MP method (Leibner-Ciszak *et al.*, 2010) revealed also only one genotype among *M. canis* strains isolated in Central Poland. It seems that the lack of intraspecies polymorphism could be due to the genetic stability of *M. canis* genome. These data are in conflict with the results obtained by Cano *et al.* (2005), who found a total of 21 genotypes among 24 human isolates of *M. canis* using ISSR-PCR (Inter-Simple-Sequence Repeat PCR) what suggested that distribution of this zoophilic dermatophyte was restricted even to a single patient.

In the present study, we have used $(GACA)_4$ and $(ACA)_5$ primer used previously by Shehata *et al.* (2008) and Cano *et al.* (2005), respectively to analyze *M. canis* strains isolated from patients and from animals, mainly from Central Poland.

* Corresponding author: A. Dobrowolska, University of Łódź, Department of Genetics of Microorganisms; 90-237 Łódź, Poland, ul. Banacha 12/16; phone: +48 42 635-47-72; **e-mail: anidobro@biol.uni.lodz.pl**

Experimental

Materials and Methods

Fungal strains. Thirty-two clinical isolates of *M. canis*, *canis*, seven strains isolated from dogs and six strains isolated from cats were used in this study (Table I). Among them, 12 were isolated from females between 2 and 54 years old, 20 were isolated from males between 3 and 64 years old. Traditional identification based on the fungus morphology observed under microscope, was performed in the Department of Dermatology and Venerology, Medical University of Łódź.

DNA extraction. Genomic DNA was extracted from a small amount of mycelium by a self-modified mini preparation method (Liu *et al*., 2000). Mycelium was suspended in 700 µl of lysis buffer (400 mM Tris-HCl, 60 mM EDTA, 150 mM NaCl, 1% sodium dodecyl sulfate), and incubated at 60°C for 1 hour. After addition of 210 µl of 3 M sodium acetate, the homogenate was centrifuged at 12 000 rpm for 10 minutes. The supernatant was successively extracted with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was treated with RNAase at a final concentration of 50 µg/ml for 20 minutes at 57°C. Than, samples were precipitated using 3 volumes of cold ethanol in the presence of 300 mM sodium acetate and DNA was centrifuged for 10 minutes. The pellet was washed with 70% ethanol and air dried. DNA was dissolved in 50 µl 1x TE buffer, 1 µl of the resulting solution was used as a template in the following PCR reaction.

Molecular identification by PCR-RFLP method. Morphological identification was confirmed by PCR-RFLP identification targeting ITS1-5.8S-ITS2 region and using *Hinf*I restriction enzyme (Dobrowolska *et al*., 2006).

$(GACA)_4$ and $(ACA)_5$ typing. PCR amplification was performed using $(GACA)_4$ and $(ACA)_5$ primers. Each PCR mixture (30 µl) contained 1 µl of genomic DNA, 1 µl of DMSO, 4 µl of 25 mM $MgCl_2$, 0.5 µl of 50 pmol $(GACA)_4/(ACA)_5$ primer, 8.5 µl of distilled water and 15 µl of MasterMixTaq Polymerase (Qiagen). PCR was performed as described previously by Shehata *et al*. (2008) and Cano *et al.* (2005). Detection of PCR products was performed by electrophoresis in 1% agarose gel stained with ethidium bromide and visualized by UV light.

Results

Molecular identification of *M. canis* isolates. The genomic DNAs of the reference strain CBS 113480 and forty-five examined *M. canis* isolates from patients and animals in Central Poland were amplified using universal primers ITS1 and ITS4 (Jackson *et al*., 1999). The size of the obtained PCR products was approximately 700 bp for all strains. We performed RFLP analysis for all 45 PCR products and the reference strain, using *Hinf*I restriction enzyme (Dobrowolska *et al.*, 2006). Additionally using computer software we generated hypothetical RFLP profiles (213, 194, 142, 108, 8 bp) based on the DNA sequence (AF 168127) of examined region. *Hinf*I digestion of ITS1-5.8S-ITS2 region revealed a pattern unique for *M. canis* (Fig. 1).

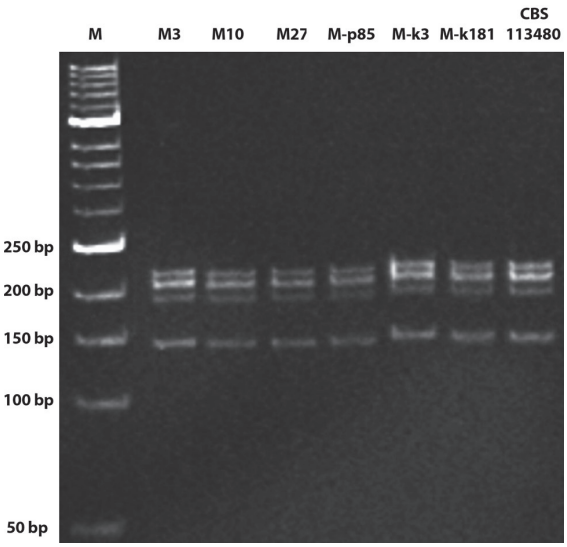

Fig. 1. Exemplary polyacrylamide-gel electrophoresis of PCR products digested with *HinfI* restriction enzyme. The ITS1-ITS4 set of primers was used to amplify ITS1-5,8S rDNA-ITS2 region. Obtained profiles correspond to *Microsporum canis*. Abbrevations above the lanes correspond to the species names assigned during traditional identification (see Table I); bp – base pair.

RAPD analysis. All of the examined *M. canis* isolates obtained from the patients and animals in Poland gave band patterns after RAPD amplification with $(GACA)_4$ and $(ACA)_5$ primer. Among them, we distinguished only one type, designated A, which was characteristic for all isolates of *M. canis* originating from Poland and for the reference strain. The $(GACA)_4$ fingerprints of 45 analyzed isolates, yielded up to 11 bands, ranging from approximately 300 to 2000 bp in length (Fig. 2) while the results obtained by Shehata *et al.* (2008) revealed profiles, with up to 11 bands but ranging from 600 bp to 2500 bp. In case of the $(ACA)_5$ fingerprints we observed two bands, approximately 500 bp, 850 bp (Fig. 3).

Discussion

Dermatomycosis induced by *M. canis* has became a serious problem in Poland in recent years. Epidemiological studies conducted in Poland between January 1,

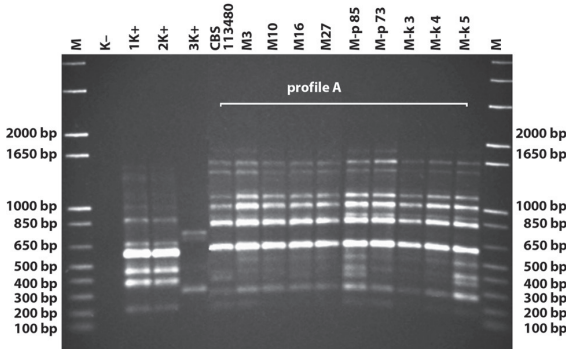

Fig. 2. Exemplary amplification profiles of DNA fragments by RAPD using repetitive primer $(GACA)_4$.

M – molecular marker 1Kb Plus, K– negative control, K+ – positive control (1 – *T. rubrum*, 2 – *T. mentagrophytes*, 3 – *E. floccosum*), profile A corresponds to *Microsporum canis* strains isolated from parients and animals in Central Poland. Abbrevations above the lanes correspond to the species names assigned during molecular identification (see Table I); bp – base pair.

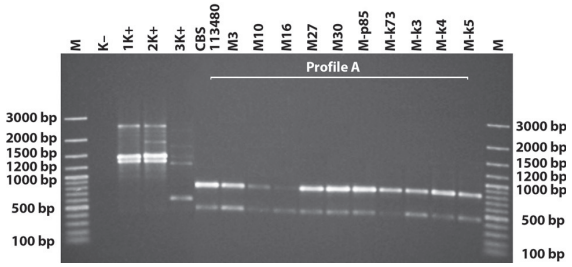

Fig. 3. Exemplary amplification profiles of DNA fragments by RAPD using primer $(ACA)_5$.

M – molecular marker 1 Kb Plus, K- negative control, K+ – positive control (1 – *T. rubrum*, 2 – *T. mentagrophytes*, 3 – *E. floccosum*), profile A corresponds to *Microsporum canis* strains isolated from parients and animals in Central Poland. Abbrevations above the lanes correspond to the species names assigned during molecular identification (see Table I); bp – base pair.

1989 and December 31, 2007 (Macura *et al*., 2008) revealed an increase in frequency of *tinea corporis* and *tinea capitis*. For example, in this period of time infections caused by *M. canis* raised from 2.64% up to 5.17%. The authors explained that the increase in *M. canis* infection resulted from buying domestic animals like cats, dogs, hamsters which can be a source of infection of this dermatophyte. The infection agents are arthrospores, which are asexual spores (present in the parasitic state of *M. canis*) formed by segmentation of the fungal hyphae. Sexual spores are absent in the parasitic phase (Sparkers *et al*., 2000).

Molecular analysis of dermatophytes revealed diversity between genus and species. *M. canis* is very unique in comparison with other dermatophytes. Typing methods based on molecular markers used in Randomly Amplified Polymorphic DNA (RAPD) (Mochizuki *et al.*, 1997; Kaszubiak *et al*., 2004; Gräser *et al*., 2000), the specific amplification of internal transcribed spacer and non-transcribed spacer regions of rDNA genes (Mochizuki *et al*., 2003; Yazdanparast *et al*., 2003) were performed, but the level of intraspecies polymorphisms within *M. canis* isolates was low.

In our study, we have utilized $(GACA)_4$ repetitive primer previously used by Faggi *et al*. (2001) and Shehata *et al.* (2008) and $(ACA)_5$ primer previously used by Cano *et al*. (2005) for differentiation of *M. canis* strains isolated from patients and animals in Central Poland. The results obtained by Faggi *et al*. (2001) showed that there was no intraspecies variability among 49 analyzed *M. canis* strains isolated from human and animals (cats and dogs) in Spain. Shehata *et al.* (2008) analyzed only 4 strains of *M. canis* isolated from Egyptian patients and among them also identified only one genotype. Our $(GACA)_4$ typing results are in agreement with those mentioned above, as we distinguished only one genotype (A) among analyzed 45 *M. canis* strains isolated from patients and animals. This can be due to the low frequency of changes in DNA among populations of analyzed strains. Results confirming this thesis have been shown by Sharma *et al.* (2007), who observed overrepresentation of one genotype (cluster I) containing 74% of the human isolates and 23% of the animals strains, which had a higher degree of virulence and had a pandemic distribution. The authors showed that some *M. canis* strains had a higher infective potential to humans but there was no association between genotypes and type of tinea caused by this zoophilic dermatophyte.

In case of different geographical origins of the *M. canis* used in the present study and those used by Shehata *et al.* (2008), the obtained profiles consisted of 11 bands but the size range was slightly different. These results may suggest that $(GACA)_4$ primer has utility for differentiation of *M. canis* strains isolated from patients and animals geographically isolated areas. However, RAPD techniques causes some problems regarding reproducibility especially when the results are compared between the laboratories. Variation in the obtained patterns can occur as a results of subtle differences in the primer or template concentration, the temperature variations during amplification or differences in magnesium concentration in the reaction mixture (Ellsworth *et al*., 1993).

Interestingly, *T. rubrum* (120358) and *T. mentagrophytes* (120357) originated from CBS (Centraalbureau voor Schimmelcultutes, Utrecht, The Netherlands) gave the same pattern using $(GACA)_4$ and $(ACA)_5$ primer (Fig. 2, Fig. 3) what may suggest that these oligonucleotides do not have very high differentiation power. On the other hand $(GACA)_4$ – based PCR revealed the presence of four profiles among *Trichophyton ajelloi* strains (geophilic dermatophyte) isolated from soil in Poland (Dobrowolska *et al*., unpublished data).

The $(ACA)_5$ – based typing of analyzed *M. canis* collection gave only one pattern (A; Fig. 3). These results

are in conflict with data obtained by Cano *et al.* (2005) who proposed ISSR-PCR (Inter Single Sequence Repeat PCR) for typing of *M. canis* isolates. Differentiation of 21 genotypes among 24 examined isolates of *M. canis* using $(ACA)_5$ and $(CCA)_5$ was very promising but the authors did not confirm traditional mycological identification with a molecular method such as *e.g.* PCR-RFLP (which was done in this study) and no reference strain was analysed. Sharma *et al.* (2007) disputed the high discriminatory power of ISSR-PCR, explaining it its low reproducibility. Other studies in which typing of *M. canis* strains was conducted, *e.g.* using RAPD, reported low variability among epidemiologically unrelated strains from cats, dogs and humans, despite their morphological diversity (Faggi *et al.*, 2001; Brilhante *et al.*, 2005).

In our study clinical isolates of *M. canis* were obtained from patients and animals from Łódź, thus it cannot be excluded that this area is dominated by the single clone of *M. canis.* However, the reference strain, which was isolated in Germany, has given the identical pattern. This problem would be probably solved after examination of the greater number of *M. canis* isolates from different regions of Poland and other countries, as well as after application of other methods such as typing method based on microsatellite markers using specific primers, $McGT_{17}$ and $McGT_{13}$, proposed by Sharma *et al.* (2007). Such analyses are actually performed by our group.

Acknowledgements

This work was supported by grant (N 303 568938) from The Ministry of Science and Higher Education. This research was also supported by the European Union within the European Social Fund and National Budget in the framework of the Operation 2.6 Integrated Operational Program of Regional Development, in connection with realization of project "Scholarships supporting innovative scientific researches of PhD students"

Literature

Brilhante R.S., R.A. Cordeiro and M.F. Rocha. 2005. Antifungal susceptibility and genotypical pattern of *Microsporum canis* strains. *Can. J. Microbiol.* 51: 507–510.

Cano J., A. Rezusta and J. Guarro. 2005. Inter-single-sequence-repeat-PCR typing as a new tool for identification of *Microsporum canis* strains. *J. Dermatol. Sci.* 39: 17–21.

Dobrowolska A., P. Stączek and M. Kozłowska. 2006. PCR-RFLP analysis of the dermatophytes isolated from patients in Central Poland. *J. Dermatol. Sci.* 42: 71–74.

Ellsworth D.L., K.D. Rittenhouse and R.L. Honeycutl. 1993. Artifactual variation in tandomly amplified polymorphic DNA banding patterns. *Biotechniques* 14: 214–217.

Faggi E., G. Pini and F. Mancianti. 2001. Application of PCR to distinguish common species of dermatophytes. *J. Clin. Microbiol.* 39: 3382–3385.

Gräser Y., A.F.A. Kuijpers and G.S. de Hoog. 2000. Molecular taxonomy of the *Trichophyton rubrum* complex. *J. Clin. Microbiol.* 38: 3329–3336.

Jackson C.J., R.C. Barton and E.G. Evans. 1999. Species identification and strain differentiation of dermatophyte fungi by analysis of ribosomal DNA intergenic spacer regions. *J. Clin. Microbiol.* 37: 931–936.

Kaszubiak A., S. Klein and Y. Gräser. 2004. Population structure and evolutionary origins of *Microsporum canis*, *M. ferrugineum* and *M. audouinii*. *Infect. Genet. Evol.* 4: 179–186.

Leibner-Ciszak J., A. Dobrowolska and P. Staczek. 2010. Evaluation of a PCR melting profiles of *Trichophyton rubrum* and *Trichophyton interdigitale. J. Med. Microbiol.* 59: 185–192.

Liu D., S. Coloe and J. Pederson. 2000. Rapid mini-preparation of fungal DNA for PCR. *J. Clin. Microbiol.* 38: 471.

Macura A.B., P. Krzysciak and M. Bochenek. 2008. Trends in the spectrum of dermatophytes causing superficial mycoses in the past decade. *Mikol. Lek.* 15: 76–79.

Meyer W., T.G. Mitchell and R. Vilgalys. 1993. Hybrydization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans. J. Clin. Microbiol.* 31: 2274–2280.

Meyer W., G.N. Latouche and T. Sorrel. 1997. Identification of pathogenic yeasts of the imperfect genus *Candida* by polymerase chain reaction fingerprinting. *Electrophoresis* 18: 1548–1559.

Mochizuki T., N. Sugie and M. Uehara. 1997. Random amplification of polymorphic DNA is useful for differentiation of several antrhopophilic dermatophytes. *Mycoses* 40: 405–409.

Mochizuki T., H. Ishizaki and E.G.V. Evans. 2003. Restriction fragment length polymorphism analysis of ribosomal DNA intergenic regions is useful for differentiating strains of *Trichophyton mentagrophytes*. *J. Clin. Microbiol.* 41: 4583–4588.

Sharma R., S. de Hoog and Y. Gräser. 2007. A virulent genotype of *Micropsorum canis* is responsible for the majority of human infection. *J. Med. Microbiol.* 56: 1377–1385.

Shehata A.S., P.K. Mukherjee and M.A. Ghannoum. 2008. Single-step PCR using $(GACA)_4$ primer: utility for rapid identification of dermatophyte species and strains. *J. Clin. Microbiol.* 46: 2641–2645.

Sparkers A.H., A. Robinson and S.E. Shan. 2000. A study of the efficacy of topical and systemic therapy for treatment of *M. canis* infections. *J. Feline Med. and Surg.* 2: 135–142.

Weitzman I. and R.C. Summerbell. 1995. The dermatophytes. *Clin. Microbiol. Rev.* 8: 240–259.

Yazdanparast A., C.J. Jackson and E.G.V. Evans. 2003. Molecular typing of *Trichophyton rubrum* indicated multiple strain involvement in onychomycosis. *Br. J. Dermatol.* 148: 51–54.