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ORIGINAL ARTICLE

The best type of inoculum for testing the antifungal drug susceptibility of *Microsporum canis*: In vivo and in vitro results

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Summary

Background: Data correlating in vitro drug susceptibility of *Microsporum canis* with clinical outcomes of its infections are lacking as well as the most suitable inoculum and incubation time in broth microdilution assays.

Objectives and Methods: *Microsporum canis* strains were collected from animal hosts that tested positive (Group I; n = 13) and negative (Group II; n = 14) to this pathogen following itraconazole (ITC) therapy. In vitro ITC susceptibility was assessed according to the Clinical Laboratory Standards Institute (CLSI M38-A2) methodology using conidia, hypha-conidia and arthroconidia at 3 and 7 days of incubation in order to assess the most suitable inoculum and incubation time. Successively, ketoconazole (KTC), voriconazole (VRC), terbinafine (TRB), posaconazole (PSZ), fluconazole (FLC) and griseofulvin (GRI) susceptibilities were assessed using the chosen inoculum.

Results: The MIC values of ITC after three-day incubation were equal than those recorded after 7-day incubation.

Itraconazole MICs were ≤1 μg/mL for strains from Group II and >1 μg/mL for those of Group II only when conidia were used. All strains showed high susceptibility to VRC, POS, TEB and low susceptibility to ITC, KTC, GRI and FLC regardless of the source and incubation time.

Conclusions and clinical importance: Results suggest that correlation between the in vitro results and clinical outcome was observed only by incubating conidia for 3 days at 30 ± 2°C. These conditions might be most suitable to assess in vitro susceptibility of *M. canis* and assist in determining the occurrence of drug resistance and cross-resistance phenomena.

KEYWORDS

antifungal susceptibility, arthroconidia, clinical resistance, conidia, dermatophytes, Hyphae, arthroconidia

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1 | INTRODUCTION

Microsporum canis is a zoophilic dermatophyte responsible for human and animal infections worldwide.[1,2] In humans, *M. canis* is associated with *tinea capitis*, *tinea corporis*, *tinea pedis* and *onychomycosis*, whilst in veterinary species, infections cause multifocal alopecia, scaling and circular lesions.[2,3] *M. canis* transmission occurs via direct contact with clinically or subclinically infected animals (mainly cats), or with spores that remain viable in the environment for up to 18 months.[4]

As *M. canis* infections are highly contagious, antifungal treatment should be systematically recommended to shorten the course of the infection, to reduce dissemination of infective material into the environment and to prevent spread to other animals and people.[2,5] Several antifungal agents were employed into clinical practice for the treatment of *M. canis* infections in veterinary and human medicine. In particular, griseofulvin (GRI), itraconazole (ITC) and terbinafine (TRB) were used in veterinary medicine and fluconazole (FLC) in human medicine.[2,5,6] The activity spectrum of these compounds is variable, and treatment failure is recorded in 25%-40% of treated human patients, as a consequence of poor patient compliance, lack of drug penetration into tissue, medication bioavailability, drug-drug interactions and/or the occurrence of antifungal resistance.[7] Particularly, the latter is considered an emerging threat involving many fungal species (ie *Aspergillus* and *Candida* spp.), but antifungal resistance in dermatophytes has been only described for *Trichophyton. rubrum*,[6,8] *Trichophyton. mentagrophytes*[9] and, rarely in *M. canis*.[10] The frequency of azole treatment failures of *M. canis* infections in animals is scant. However, recently a study reported TER treatment failure in *M. canis* infections of animals.[10] Nevertheless, these observations have led to an increased interest in antifungal susceptibility testing for dermatophytes. However, till date, methodological inconsistencies mainly related to the standardisation of the inoculum preparation and incubation times impair unequivocal interpretations of in vitro susceptibility assays.[11] Several studies have been conducted on antifungal susceptibility of *M. canis* using inocula consisting of both conidia and hyphae-conidia, and different incubation times (from 3-7 days).[11] In some dermatophytes (*T. rubrum* and *T. mentagrophytes*), the wall of the macroconidia is considerably thicker than the hyphae, thus affecting the overall antifungal susceptibility profile of the fungus.[12,13] However, dermatophytes also produce arthroconidia, a cellular structure presumably more resistant to antifungals, which may be responsible for therapeutic failure.[7,14] No data are currently available on the antifungal susceptibilities of these three fungal structures (ie conidia, hyphae-conidia and arthroconidia) of *M. canis*. Since data correlating in vitro drug susceptibility with clinical outcomes are lacking the information on the most suitable inocula and incubation time to use in antifungal broth microdilution, susceptibility assays for *M. canis* were never assessed. Therefore, the aims of this study were to i) determine the in vitro ITC susceptibility of *M. canis* conidia, hyphae-conidia and arthroconidia following incubation for 3 and 7 days and ii) compare the ITC MICs of *M. canis* strains obtained from animals that tested positive and negative to the fungus following ITC therapy in order to assess the most suitable inoculum and incubation time to use in antifungal broth microdilution susceptibility assays for *M. canis*. Using the best condition, in this study the antifungal susceptibility of *M. canis* to ketoconazole (KTC), FLC, posaconazole (POS), voriconazole (VRC), GRI and TRB and iv) the occurrence of a probable drug resistance or cross-resistance phenomena were also assessed.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to.

2.2 | *Microsporum canis* strains

Hair and skin scraping samples were taken from the skin lesions of animals using a sterile lancet or pliers.[15] A total of 27 selected clinical strains of *M. canis* were divided into two groups according to the clinical outcome to once daily dose of ITC oral therapy. Group I included 13 strains from 13 dogs treated with ITC (10 mg/kg) that tested positive to this fungus following a consecutive 3-monthly oral therapy. Group II included 14 *M. canis* strains from dogs (12) and cats (2) treated with ITC (10 mg/kg) following a consecutive 3-monthly oral therapy that subsequently tested negative to this fungus. The strains were identified based on colonial morphology and microscopic features of the hyphae, macroconidia and microconidia,[16] and were molecularly identified by an improved molecular diagnostic assay as previously reported.[17] Isolates were stored at −80°C at the Department of Veterinary Medicine, University of Bari (Italy). Prior to testing, each strain was sub-cultured at least twice onto Potato Dextrose agar (PDA, Liofilchem, Roseto degli Abruzzi, Italy) plates at 30°C for 10 days to ensure strain purity and viability.

2.3 | Medium for antifungal test

A broth microdilution assay was performed in RPMI 1640 medium (Sigma-Aldrich, Milano, Italy) with L-glutamine but without sodium bicarbonate and buffered with 0.165 mol/L morpholine propane sulphonic acid (MOPS) (Sigma-Aldrich) at pH 7.0.

2.4 | Antifungal agents

The following drugs were obtained in their standard powder state: TRB and GRI (Sigma-Aldrich, Milan, Italy), KTC and VRC (Novartis, Basel, Switzerland), FLC (Pfizer), ITC (Janssen Research Foundation, Beerse, Belgium) and PSZ (Schering Plough Research, NJ, USA). Stock solutions of FLC (10 mg/mL), KTC (10 mg/mL), ITC (10 mg/mL), VRC (10 mg/mL), PSZ (10 mg/mL), TRB (10 mg/mL) and GRI (50 mg/mL) were prepared by dissolving the powders in their respective solvents. FLC was dissolved in distilled water, whilst the other

compounds were dissolved in 100% dimethyl sulphoxide (DMSO, Sigma-Aldrich). The stock solutions were stored at −20°C until use.

2.5 | Inoculum preparation

Three types of inocula (ie conidia, hyphae-conidia and arthroconidia) were prepared (Figure 1). Hyphae-conidia and conidial suspensions were obtained from 14-day-old *M. canis* cultures on PDA incubated at 28°C. For arthroconidial suspensions, *M. canis* was cultured onto 2% yeast extract + 1% peptone agar at 12% CO_2 at 30 ± 2°C for 21 days as previously described.[7] Mature colonies were submerged with approximately 3 mL of sterile saline solution (0.85% w/v), and the surface was scraped with the tip of a Pasteur pipette. The resulting mixture was transferred into 5-mL sterile tubes.

For hyphae-conidial suspension, heavy particles of mixture were allowed to sediment for 15 minutes at room temperature. The supernatant was transferred into another sterile tube and adjusted to an optical density of 2.4 McFarland using a turbidimeter (DEN- 1 McFarland Densitometer, Biosan) which is equivalent to 1- 5 × 10^6 colony forming units (CFU)/mL), as inferred by quantitative plate counts of CFU in PDA.

For conidial suspension, the mixture of conidia and hyphal fragments were allowed to sediment for 15 minutes at room temperature and the supernatant was collected and filtered using sterile filter paper (Whatman filter model 40, pore size, 8 μm), which retains hyphal fragments.[18] The density of the filtered suspension was adjusted to an optical density of 2.4 McFarland as inferred above.

The resulting mixture of arthroconidia was agitated for 1h at 25°C. The supernatant was filtered using sterile filter paper (Whatman filter model 40, pore size, 8 μm). The density of the filtered suspension was adjusted to an optical density of 2.4 McFarland as reported above.

2.6 | In vitro susceptibility testing

The antifungal susceptibility of the *M. canis* inocula to ITC was tested using the reference CLSI BMD assay[19] with some modifications. Antifungal drug stocks and the inoculum suspensions were prepared as described above.

The concentration of each antifungal drug ranged from 0.008 to 16 μg/mL, with the exception of FLC and GRI, whose concentration ranged from 0.06 to 64 μg/mL. Visual reading of plates was performed after 3 and 7 days of incubation, respectively, at 30 ± 2°C. The MIC of each strain was defined as the lowest concentration of the agent producing a predominant decrease in turbidity (ie 100% of inhibition) when compared to the control growth, as previously described.[20,21] Each plate was run in triplicate, and each drug dilution was tested in duplicates in each plate. Quality control strains (*Candida parapsilosis* ATCC 22 019 and *Candida krusei* ATCC 6258; American Type Culture Collection) were included to ensure accuracy of the drug dilutions and reproducibility of results.[19]

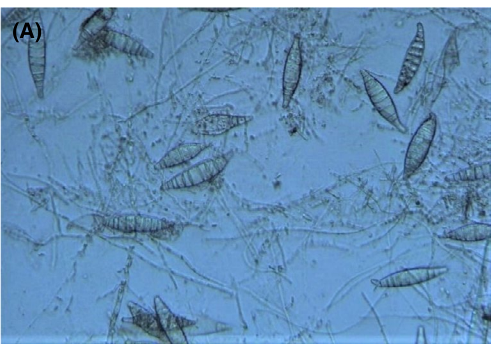
The results were reported as MIC range, MIC mean with standard deviation, MIC50 (MICs at which 50% of the strains were inhibited) and MIC90 (MICs at which 90% of the strains were inhibited).

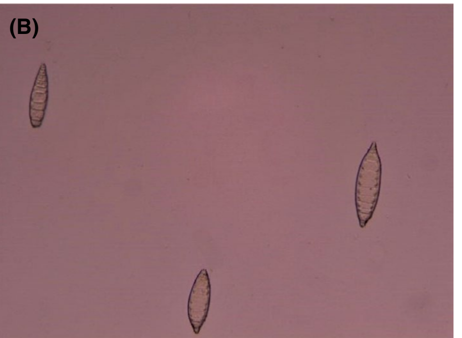
2.7 | Statistical analysis

Student's *t* test was used to evaluate the differences among MIC mean values of different antifungal drugs within and between Groups I and II. A value of $P \leq .05$ was considered statistically significant.

3 | RESULTS

All quality control MIC values were within the ranges established by the CLSI.[19] The ITC MIC values of *M. canis* strains from animals of Group I and II obtained using different inocula and incubation times are reported in Table 1. The MIC values of ITC recorded after 3-day incubation were equal or marginally lower ($P > .05$; Table 1) than those recorded after seven-day incubation. The ITC MIC values obtained with arthroconidia were significantly lower than those recorded by using conidia and hypha-conidia, regardless of incubation time and *M. canis* source (Table 1, $P < .05$). The ITC MIC values (MIC_{50}, MIC_{90}, mMIC) of hypha-conidia and conidia varied according to *M. canis* source being higher for strains from Group I. The lowest





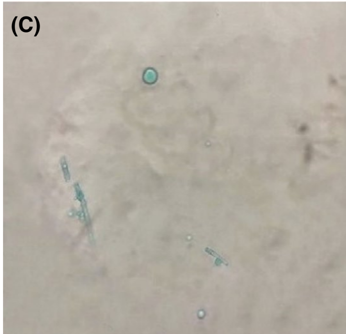


FIGURE 1 Hyphae-conidia (A), macroconidia (B) and arthroconidia (C) inocula used for in vitro antifungal susceptibility of *Microsporum canis* at ×40 magnification

TABLE 1 Itraconazole MIC values (μg/mL) of *Microsporum canis* from Group I (animals with a negative clinical outcome) and Group II (animals with a positive clinical outcome) using hyphae-conidia, conidia and arthroconidia at 3 and 7 days of incubation

			Hyphae and conidia				Conidia				Arthroconidia			
Groups	N. Strains	Reading time	Range	MIC mean (SD)	MIC_{50}	MIC_{90}	Range	MIC mean (SD)	MIC_{50}	MIC_{90}	Range	MIC mean (SD)	MIC_{50}	MIC_{90}
Group I	13	3 days	1-16	4.4 (5.2)[f]	2	4	0.5-16	4.5 (5.2)[a,e]	2	8	0.25-4	1.5 (1.4)[a,i]	0.5	2
		7 days	1-16	5.5 (5.9)[b,g]	2	8	0.5-16	5.4 (5.9)[c,h]	2	8	0.008-8	1.8 (2.2)[b,c]	0.5	2
Group II	14	3 days	0.008-1	0.8 (0.5)[d,f]	1	1	0.008-1	0.4 (0.3)[d,e]	0.5	0.5	0.008-1	0.5 (0.4)[i]	0.5	1
		7 days	0.008-2	1.1 (0.7)[g]	1	2	0.008-1	0.7 (0.5)[h]	1	1	0.008-2	0.8 (0.6)	1	1

[a-i]Students *t* test—statistically significant differences ($P \le .05$) are marked with the same letters.

value of ITC MIC values in Group II samples was recorded using conidia and three-day incubation (Table 1).

The ITC MIC values of conidia were ≤1 μg/mL for strains from Group II and >1 μg/mL for those of Group I. All the *M. canis* strains showed low MIC values to VRC, PSZ and TRB, and high MIC values to ITC, KTC, GRI and FLC, regardless of the source of collection and incubation time (Table 2). The MIC mean value for all compounds tested in Group I was higher than those from Group II. High FLC (>64 μg/mL) MICs was recorded using the strains that showed high ITC MIC values (>1 μg/mL).

4 | DISCUSSION

Results of this study suggest that the type of inocula used in in vitro antifungal susceptibility assays of *M .canis* affects the ITC MIC values, as previously reported for *T. mentagrophytes* and *T. rubrum*.[22] In addition, the ITC antifungal susceptibility observed for samples obtained from animals infected by *M. canis* and that had tested positive (Group I) or negative (Group II) to this pathogen following ITC therapy was compared, thus allowing us to evaluate the most suitable type of inocula or incubation time for in vitro susceptibility testing. No differences were observed in the MIC values obtained after 3 and 7 days, thus supporting the hypothesis that the time of incubation does not affect the ITC MIC values as previously observed for *T. rubrum*.[8,22,23] Therefore, we suggest that a 3-day incubation is optimal in in vitro antifungal susceptibility tests of *M. canis*, which considerably shortens the time needed to obtain reliable diagnostic results. ITC MIC values observed in in vitro antifungal susceptibility tests varied according to the inocula used, with arthroconidia displaying the highest susceptibility than the other structures tested, and hyphae-conidia the lowest. In contrast, arthroconidia of *T. rubrum*, *T. tonsurans* and *T. equinum* are less susceptible to antifungals,[7] which is possibly linked to their thick conidia walls.[24] The MIC values observed using hyphae-conidia were higher than those recorded using conidia alone, particularly in comparative analyses of MIC values of *M. canis* obtained from Groups I and II, respectively. Interestingly, a correlation between the antifungal in vitro results and clinical outcome was observed only by incubating conidia for three days at 30 ± 2°C.

Particularly, ITC MIC values lower and higher than 1 μg/mL was observed using *M. canis* conidia from Group II and Group I, respectively, thus suggesting that antifungal resistance may occur in *M. canis* strains from animals that tested negative to this pathogen following in vivo ITC therapy (Group I). Therefore, the in vitro results of MIC >1 μg/mL correlates well with the in vivo results of a negative clinical outcome following a consecutive 3-monthly ITC oral therapy, thus suggesting a probable resistance phenomena to ITC for *M. canis*.

Interestingly, ITC MIC >1 μg/mL represents the epidemiological cut-off (ECV) for filamentous fungi (i.e. *Aspergillus* spp.) and the clinical breakpoint (CBP) for yeasts (i.e. *Candida* spp.).[25,26] Accordingly, high ITC or FLC MIC values for *M. canis* strains were previously

TABLE 2 Itraconazole (ITC), ketoconazole (KTC), voriconazole (VRC), posaconazole (POS), terbinafine (TRB), fluconazole (FLC) and griseofulvin (GRI) MIC (μg/mL) data of *Microsporum canis* from Group 1 and Group II at 3 and 7 d of incubation using conidia

			Conidia at 3 d of incubation				Conidia at 7 d of incubation			
Groups	Number	Drugs	MIC Range	MIC mean (SD)	MIC_{50}	MIC_{90}	MIC Range	MIC mean (SD)	MIC_{50}	MIC_{90}
Group I	13	ITC	0.5-16	4.5 (5.2)[a]	2	8	0.5-16	5.4 (6)[d]	2	8
		KTC	0.125-4	1.4 (1.2)	1	2	0.125-4	1.4 (1.26)	1	2
		VRC	0.008-1	0.14 (0.27)	0.03	0.125	0.016-1	0.14 (0.28)	0.03	0.125
		PZ	0.008-2	0.5 (0.6)[b]	0.25	1	0.008-2	0.5 (0.60)	0.25	1
		TRB	0.008-0.25	0.07 (0.09)	0.016	0.25	0.008-0.25	0.07 (0.10)	0.016	0.25
		FLC	4-128	36.9 (44.0)[c]	8	64	4-128	36.9 (44.0)[e]	8	64
		GRI	0.125-2	1.0 (0.72)	0.5	2	0.125-2	1.0 (0.72)	0.5	2
Group II	14	ITC	0.008-1	0.4 (0.3)[a]	0.5	0.5	0.008-1	0.7 (0.5)[d]	1	1
		KTC	0.008-4	0.7 (1.0)	0.25	1	0.008-4	1.2 (1.4)	0.5	2
		VRC	0.008-0.125	0.03 (0.03)	0.016	0.06	0.008-0.125	0.03 (0.03)	0.016	0.06
		PZ	0.008-1	0.1 (0.30)[b]	0.03	0.06	0.008-1	0.1 (0.3)	0.03	0.25
		TRB	0.008-0.5	0.08 (0.14)	0.016	0.125	0.008-0.5	0.13 (0.14)	0.125	0.25
		FLC	0.06-32	7.9 (10.5)[c]	4	8	1-32	8.2 (10.4)[e]	4	8
		GRI	0.03-2	0.6 (0.51)	0.5	1	0.03-2	0.6 (0.52)	0.5	1

[a-j]Students *t* test—statistically significant differences ($P \le .05$) are marked with the same letters.

recorded suggesting the occurrence of resistance phenomena although the correlations between the in vitro antifungal results and clinical outcome were never recorded.[27]

Our study supports the validity of the CLSI guidelines.[28-30] However, these guidelines recommend separation of the fungal structures (hyphae and conidia) through sedimentation for 15-20 minutes, and the use of the upper part of the suspension for susceptibility testing.[30] The results of our study show that the sedimentation of the inoculum is inefficient for separating dermatophyte hyphae, and filtration might be required, similarly to *T. rubrum* and *T. mentagrophytes*.[18] Using these test conditions, the most effective drugs against *M. canis* strains obtained from Groups I and II were VRC, PSZ and TRB, thus confirming previous reports.[20,31] The highest FLC MICs (ie MIC > 64 μg/mL) were recorded using the strains with high ITC (MIC > 1μg/mL), thus suggesting the occurrence of cross-resistance.

In conclusion, data herein reported suggest the importance of the inoculum type and incubation time in determining the in vitro antifungal susceptibility of *M. canis* using micro dilution assays. Based on our data, conidia and three-day incubation at 30 ± 2°C are most suitable for testing in vitro antifungal susceptibility of *M. canis*. The above conditions may also assist in determining the occurrence of probable drug resistance phenomena. Whilst the suitability of these test conditions must be tested in further studies conducted in other laboratories to ensure reproducibility, data from our work represent a solid foundation for the development of standardised antifungal susceptibility tests for *M. canis*. Furthermore, complementary studies on *M. canis* resistance are advocated in order to investigate the molecular mechanisms of this phenomenon.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

C.C and ACI conceptualised the study and wrote the manuscript. PC performed the clinical evaluation of animals and collected the samples. WR, ACI and C.C performed the experimental trial. C.C contributed to interpretation of the study data. C.C, C. CZ and D.O revised, edited and made intellectual inputs in the manuscript. All authors have read, revised and approved the final version of manuscript.

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