Dermatophytes develop resistance to the monoterpenes geraniol and citronellol

Desarrollo de resistencia de los dermatofitos a los monoterpenos geraniol y citronelol

Gustavo Nunes Cardoso[1] https://orcid.org/0000-0001-6872-0029
Kaltz Victor Souza Silva[1] https://orcid.org/0000-0003-4786-9734
Maria Islaine de Oliveira Lima[1] https://orcid.org/0000-0003-1076-6467
Juliana Moura Mendes Arrua[2] https://orcid.org/0000-0001-5483-3612
Fillipe de Oliveira Pereira[1*] https://orcid.org/0000-0002-3081-4174

[1]Universidade Federal de Campina Grande, Laboratório de Bioquímica, Unidade Acadêmica de Saúde, Centro de Educação e Saúde. Cuité, Brasil.
[2]Universidad Nacional de Asunción Laboratorio de Biotecnología, Centro Multidisciplinario de Investigaciones Tecnológicas, Dirección General de Investigación Científica y Tecnológica. San Lorenzo, Paraguay.

[*]Autor para la correspondencia: fillipeopereira@ufcg.edu.br

ABSTRACT

Introduction: Dermatophytoses are fungal infections whose treatment has been the subject of much concern throughout the world. The emergence of antifungal resistance (azoles) in clinical therapeutics is well known. However, few studies demonstrate the capacity of dermatophytes to develop resistance against natural products such as terpenes. The monoterpenes geraniol and citronellol have recognized antifungal potential and are found in various essential oils.

Objectives: To investigate the capacity of *Trichophyton rubrum*, *Microsporum canis*, and *Microsporum gypseum* to acquire resistance against citronellol and geraniol.

Methods: The minimum inhibitory concentration of the tested drugs was determined by microdilution. The fungal strains were subjected to eight successive subcultures in Sabouraud dextrose agar containing the monoterpenes in sub-inhibitory concentrations.

After this period of adaptation, the susceptibility profile to drugs was assessed by microdilution. Finally, after eight passages in culture medium without the drugs, resistance stability was again evaluated by microdilution test.

Results: From the initial citronellol and geraniol minimum inhibitory concentration values we observed an increase. In addition, a cross over effect was observed against the azole compounds. Finally, the fungi reversed their profiles of resistance against the natural drugs and showed no reversal in resistance to azoles. Although in dermatophytes the development of resistance to monoterpenes was observed, the phenomenon was not stable, as was observed against the azole drugs.

Conclusions: Citronellol and geraniol were active against resistant isolates even after dis-habituation. Thus these monoterpenes present themselves as potential therapeutic alternatives with fewer complications in the emergence of resistance.

Keywords: resistance; antifungal; habituation; terpenes; azoles.

RESUMEN

Introducción: Las dermatofitosis son infecciones fúngicas cuyo tratamiento es motivo de preocupación en todo el mundo. En la terapéutica clínica, la aparición de la resistencia antifúngica (azoles) es muy conocida. Sin embargo, pocos estudios han demostrado la capacidad de los dermatofitos de desarrollar resistencia a productos naturales como terpenos. Los monoterpenos geraniol y citronelol tienen un reconocido potencial antifúngico y se encuentran en diversos aceites esenciales.

Objetivo: Investigar la capacidad de *Trichophyton rubrum*, *Microsporum canis* y *Microsporum gypseum* para desarrollar resistencia a los monoterpenos geraniol y citronelol.

Métodos: Se determinó, mediante microdilución, la concentración inhibitoria mínima de las drogas sometidas a ensayo. Las cepas fúngicas fueron sometidas a ocho subcultivos en agar a base de dextrosa Sabouraud que contenía concentraciones subinhibitorias de los monoterpenos ensayados. Pasado este periodo de adaptación, se evaluó el perfil de susceptibilidad por microdilución. Finalmente, después de ocho pases en medio de cultivo sin las drogas, se evaluó la estabilidad de la resistencia nuevamente mediante la prueba de microdilución.

Resultados: Se observó un incremento a partir de la concentración inhibitoria mínima inicial del citronelol y geraniol, además del efecto cruzado frente a los compuestos azólicos. Finalmente, los hongos revertieron su perfil de resistencia frente a las drogas naturales, sin

mostrar resistencia a los azólicos. Aunque se observó el desarrollo de resistencia a los monoterpenos en los dermatofitos, el fenómeno no fue estable como el demostrado frente los fármacos azólicos.

Conclusiones: El citronelol y el geraniol fueron activos contra asilados resistentes incluso con posterioridad a la deshabituación. De este modo, estos monoterpenos se presentan como posibles alternativas terapéuticas y con pocas complicaciones relacionadas con la aparición de la resistencia.

Palabras clave: resistencia; antifúngicos; habituación; terpenos; azoles.

Recibido: 01/06/2019

Aceptado: 25/01/2020

Introduction

Dermatophytoses are fungal infections in keratinized tissues such as nails, hair and the stratum corneum of the skin, caused by fungi called dermatophytes which, comprise the genera *Microsporum*, *Trichophyton* and *Epidermophyton.*[1] Dermatophytes infect both males and females of all ages and have a worldwide distribution. However, hot and humid climates as found in tropical and subtropical areas contribute to the large numbers of cases in Latin America, Africa, and Asia.[2,3]

Dermatophytoses can be treated topically or systemically or using drug associations usually reserved for more complicated or chronic cases; or for patients with *Tinea unguium* or *Tinea capitis.*[4] Certain agents are used clinically to treat dermatophytosis, among which are ketoconazole imidazole and triazole compounds i.e. itraconazole and fluconazole.[5] Although it seems that the number of antifungal drugs available in the market is large, they group simply into a few chemical classes, and thus often present a restricted spectrum of action. Antifungal treatments often fail because dermatophytosis is frequently caused by *T. rubrum*, associated with high relapse and drug resistance, and like other micro-organisms; fungal cells have great capacity to develop resistance to antifungal drugs.[1]

Natural products are emerging as a both great alternative therapies, and as one of the most successful strategies for discovering new molecular candidates for future drugs.[6] Essential oils are complexes of volatile compounds found in various parts of plants. They are widely

used and constantly sought due to the strong demand for natural constituents to treat diseases.[7] Monoterpenes deserve to be highlighted. Geraniol and citronellol are alcoholic monoterpenes found in the essential oils of aromatic and medicinal plants such as *Cymbopogon winterianus* (poaceae),[8] and others.[9,10]

The repellant[11] and antimicrobial[12] activity of monoterpenes has already been highlighted. We therefore investigated their effect on the resistance profiles of *T. rubrum*, *M. canis*, and *M. gypseum* against azole drugs such as ketoconazole, fluconazole, and itraconazole using sub-inhibitory concentrations of citronellol and geraniol (to induce antifungal resistance).

Methods

Drugs

Citronellol, geraniol, ketoconazole, fluconazole and itraconazole were purchased from Sigma-Aldrich® (Brazil). Emulsions were freshly prepared for the tests by dissolving first in dimethylsulfoxide (DMSO), and sterilized distilled water to obtain a concentration of 1024 µg/mL. From this concentration, dilutions were performed to achieve a concentration of 1 µg/mL using RPMI 1640 medium

Fungi

The fungal strains *T. rubrum* LM 305 (skin), *M. canis* LM 216 (scalp) e *M. gypseum* LM 305 (skin) were taken from the culture collections of the (Federal University of Paraíba) Laboratory of Mycology. The fungi were grown in potato dextrose agar (Difco®) at 28° C for 7 days to obtain the fungi inocula in sterile saline (0.9 % NaCl). Turbidity of the final inocula was adjusted to 10^6 conidia/mL, at a wavelength of 520 nm, and transmission adjusted to 70 % in a UV-5100 Spectrophotometer.[13]

Minimum inhibitory concentration (MIC)

MIC values of the drugs-test were determined against dermatophyte fungi by microdilution technique using 96 well flat bottom micro-titer plates.[13,14] To each row of the plate was added 100 µL of the diluted test drugs in RPMI 1640. To each well of the plate was added 100 µL of a previously prepared inoculum diluted in RPMI 1640 at a ratio of 1:50. A fungal control was performed by replacing the test drug using sterile saline (growth control). A sterility control and DMSO were also performed. The plates were sealed and incubated at 28° C for 7 days. MIC was the lowest concentration of drugs capable of inhibiting observed

fungal growth in the wells by 100%. The assays were performed in triplicate a nd the MIC modal values expressed as geometric means of the results.

Induction of antifungal resistance

Test tubes containing Sabouraud dextrose agar with citronellol and geraniol at 1/2MIC and (controls) free of drugs, were inoculated with a mycelial fragment of 4 mm^2 from each fungal strain, newly-grown on potato dextrose agar. The tubes were incubated at 28°C for 5 days. This procedure was repeated 8 times under the same conditions. The final cultures were used for the preparation of the respective inoculates with saline solution. Afterwards, the new sensitivity profile of these strains (adapted) was examined; determining the MICs for geraniol and citronellol by microdilution.[15] Development of resistance was observed as an increase in the MIC value in relation to the baseline value.[16] The assays were performed in triplicate and the MIC values expressed as geometric means of the results.

Cross-resistance assessment

To verify sensitivity profiles against ketoconazole, fluconazole and itraconazole, the dermatophyte strains were first treated with sub-inhibitory concentrations of monoterpenes.[13,14] A micro-organism control was also performed in parallel in RPMI 1640, where no azole drugs were added. Thus, we would be able to compare the new fungal sensitivity profiles, and also confirm if cross-resistance to azole drugs had occurred as a result of adaptation to monoterpenes in these dermatophyte strains.

Evaluation of the resistance stability

To evaluate the stability of both direct resistance and cross resistance to the antifungals, a mycelial fragment of 4 mm^2 of the resistant strains was placed on Sabouraud dextrose agar surfaces in tubes free of drugs. The tubes were incubated at 28 °C for 5 days. This procedure was repeated 8 times. The final cultures were used for preparation of the respective inoculant in saline solution. Finally, we analyzed the new sensitivity profile of these adapted strains using microdilution. Thus, it was possible to confirm whether or not there had been any reversal in dermatophyte resistance to the test drugs.[17]

Results

Initially, the MICs of ketoconazole, fluconazole, itraconazole and the citronellol and geraniol monoterpenes against the strains (*T. rubrum* LM 305, *M. canis* LM 216 and *M.*

gypseum LM 305) were determined. The results are demonstrated in table 1. The dermatophytes were most resistant to fluconazole, since growth was inhibited only by a greater concentration of the drug (2 µg/mL). Citronellol and geraniol presented their best activity against *T. rubrum* LM 305 at MIC = 64 µg/mL.

Table 1 - Minimum inhibitory concentration (MIC) values of drugs-test against dermatophytes strains

Drugs	***Trichophyton rubrum* LM 305**	***Microsporum canis* LM 216**	***Microsporum gypseum* LM 305**
Citronellol	64	256	256
Geraniol	64	128	128
Ketoconazole	1	1	1
Fluconazole	2	2	2
Itraconazole	1	1	1

* MIC: µg/mL. Modal values of three experiments.

The fungal strains (in the crop series) were thus subjected to sub-inhibitory treatment concentrations of citronellol and geraniol (antifungal pressure); cultivated in both the presence and absence of drugs, and to respective determinations of their MIC values (Table 2). In mediums free of drugs (controls), for all strains tested, there were no changes in the MIC values in relation to the initial states (data not shown).

Table 2 - Sensitivity of dermatophyte strains after induction of antifungal resistance in the presence of citronellol and geraniol and reversal of resistance

Drugs	**Induction of resistance in a citronellol medium/Reversal of resistance***			**Induction of resistance in a geraniol medium/Reversal of resistance***		
	***Trichophyton rubrum* LM 305**	***Microsporum canis* LM 216**	***Microsporum gypseum* LM 305**	***Trichophyton rubrum* LM 305**	***Microsporum canis* LM 216**	***Microsporum gypseum* LM 305**
Citronellol	256/64	512/64	512/128	256/128	128/64	128/128
Geraniol	128/128	256/64	128/128	256/64	256/64	256/128
Ketoconazole	1/4	2/2	4/4	1/8	2/1	4/16
Fluconazole	4/8	16/32	16/16	2/4	16/32	16/8
Itraconazole	1/2	2/16	2/4	1/16	2/16	2/2

* Minimum inhibitory concentration (MIC): µg/mL. Modal values of three experiments.

The *T. rubrum* strain LM 305, after citronellol antifungal pressure, showed an increase in its MIC values (resistance) against citronellol. There was also a change in its MIC values against fluconazole and geraniol, (cross-resistance). For geraniol, the MIC also changed from 64 µg/mL to 256 µg/mL; however, this geraniol adapted strain showed no cross-resistance against the azoles, only against citronellol.

After antifungal pressure processing, *M. canis* strain LM 216 developed resistance to both monoterpenes, and, the MIC values for ketoconazole, fluconazole and itraconazole increased, presenting cross-resistance, this after treatment with either geraniol or citronellol. While treatment of this strain with citronellol induced cross-resistance to geraniol, interestingly, when treated with geraniol, this same strain became more sensitive to citronellol.

M. gypseum LM 305 also developed resistance to geraniol and citronellol after application of antifungal pressure. In relation to the emergence of cross-resistance to azoles, the strain showed behavior similar to *M. canis* LM 216, and, the treatment of this strain with citronellol did not induce cross-resistance to geraniol. However, when treated with geraniol, the strain became more sensitive to citronellol.

After the tested strains acquired resistance, it was necessary to verify the influence of subsequent dis-habituation cycles (for monoterpene action) and the reversibility of direct and cross fungal resistance to antifungals. The results are expressed in table 2. One can see that the resistance developed against citronellol was not permanent; with the exception of the MIC for geraniol against *T. rubrum* LM 305 (already citronellol resistant), indicating cross-resistance stability. Similarly, this occurred with the MICs of the natural monoterpene products in the strains that were resistant to geraniol; the majority also reversed their MICs. *T. rubrum* alone displayed stabilization of cross-resistance to citronellol. However, the MICs of the azole drugs when retested, (both when treated with citronellol or geraniol), did not indicate resistance reversal, except for *M. canis* LM 216 resistant to geraniol; (against ketoconazole).

Discussion

Mechanisms of antifungal activity of monoterpenes have been reported in the literature. Against strains of *T. rubrum*, previous studies have suggested that inhibition of ergosterol biosynthesis is the likely mechanism of antifungal activity for citronellol and geraniol.[12]

However, the literature lacks reports on the resistance of dermatophytes against natural products, including monoterpenes. The present study sought to know if *in vitro* resistance development is possible, and also if monoterpenes can yield cross resistance to azole compounds since they both share the same mechanisms of antifungal activity.

Reports in the literature concerning acquired resistance in dermatophyte fungi against essential oils and their phytochemicals are scarce. Osborne *et al*.[18] investigated acquired resistance to terbinafine in *T. rubrum* strains when cultivated with sub-inhibitory concentrations of the drug. More recently, a study by Hryncewicz-Gwo'z'dz' *et al*.[15] investigated *T. rubrum* resistance development when subjected to antifungal pressure using fluconazole and itraconazole. In this study, the authors report that sequential passages fluconazole or itraconazole against clinical isolates of *T. rubrum* resulted (in the majority of isolates) in MIC increases for both drugs. This clearly indicates the ability of *T. rubrum* to acquire resistance through exposure to sub-inhibitory drug concentrations.

The mechanisms of resistance to azole derivatives seen in *T. rubrum* and other dermatophyte species are very little understood, yet certain biochemical mechanisms have been reported. One proposed mechanism to explain *T. rubrum* resistance to azole drugs is effluxing, and an increase in the expression of efflux pumps. The *Trichophyton interdigitale* pleiotropic drug resistance gene *pdr1* (previously named *mdr1*, and *mdr2*), encodes different ABC transporters that are over-expressed when in the presence of the various antifungal drugs.[19] Another mechanism that may be involved in resistance to azole compounds is increasing modulation of lanosterol 14-alpha-demethylase, the molecular target for azole compounds. After exposure to sub-inhibitory concentrations of ketoconazole, overexpression of the *erg11* gene (which encodes the enzyme) was evidenced in *T. rubrum*.[3]

It was observed in this study that sequential passages of clinical *T. rubrum*, *M. canis* and *M. gypseum* isolates in presence of citronellol and geraniol resulted in MIC increases for citronellol, geraniol, and the azole drugs tested. This is the first report of resistance to natural products acquired by dermatophytes. This finding is of the utmost importance, since resistance modulation *in vivo* is also possible and may cause therapeutic failures.

According to Santos *et al*,[20] this *in vitro* observation has its reflections *in vivo*; and is supported by the increase in MIC values for azole agents, including itraconazole and ketoconazole in strains of *T. rubrum* obtained from patients with onychomycosis, after treatment with ketoconazole or a combination of itraconazole and terbinafine. The results also point to the development of cross-resistance, both to the azole test drugs and to the

monoterpenes. This is probably because both present similar toxic mechanisms to the fungal cell.

The stability and reversibility of direct and cross fungal resistance to antifungals are parameters that must also be analyzed due to their clinical implications. Borst *et al*.[21] cultivated isolates of *C. glabrata* with acquired resistance to fluconazole in a drug-free medium for 122 days. The resistance phenotype was lost was when subcultures in liquid medium were changed to solid. This is an important correlation for our results, since the subcultures were performed directly on solid mediums and resistance phenotype loss was also observed.

Pippi *et al*[22] investigating resistance stability showed that for isolates of resistant *C. parapsilosis*, the resistance phenotype was preserved even after subculture in the fluconazole free medium. By this, it can be inferred in relation to natural products that biochemical changes in the fungal cells occurred only in the presence of citronellol and geraniol stressor agents. This highlights an advantage for these natural products, since azole resistance plays an important role in therapeutic failures and consequently contributes to the persistence and chronicity of infections.

Several theories try to explain how genetic changes causing resistance to drugs emerge, and become established in fungal populations. Biochemical mechanisms involved in the acquisition of resistance by dermatophyte fungi, may explain both irreversible as well as reversible mutations.[23] An organism with the ability to modulate its rates of spontaneous mutation and recombination, keeping them low during conditions of low stress and increasing them in conditions of high stress, has a selective advantage over organisms with constitutively constant (high or low) rates of mutation and recombination.[24]

From the results presented in this study, one may conclude that dermatophytes develop resistance when submitted to successive sub-inhibitory concentration treatments of citronellol and geraniol. This resistance may also lead to the emergence of resistance to other drugs such as ketoconazole, fluconazole and itraconazole. These results are new, and place natural drugs, including monoterpenes as therapeutic agents susceptible to the mechanisms of resistance developed by pathogenic fungi. It was observed that resistance to these natural products is reversible.

In conclusion, our results reveal that citronellol and geraniol as having great potential as future therapeutic agents; yielding fewer complications in the emergence of resistance. Although controlled clinical studies are needed to define their true effectiveness, these

monoterpenes were active against resistant isolates even after dis-habituation, which did not occur with the majority of the azoles.

Acknowledgements

The authors thank the Mycology Laboratory, Federal University of Paraíba for the supply of fungal strains.

Bibliographic references

1. Peres NTA, Maranhão FCA, Rossi A, Martinez-Rossi NM. Dermatophytes: host-pathogen interaction and antifungal resistance. Rev Bras Dermatol. 2010;85(5):657-67.

2. Bhagra S, Ganju AS, Kanga A, Sharma NL, Guleria RC. Mycological pattern of dermatophytosis in and around ShimLa hills. Indian J Dermatol. 2014;59(3):268-70.

3. Faure-Cognet O, Fricker-Hidalgo H, Pelloux H, Leccia MT. Superficial fungal infections in a French teaching hospital in Grenoble area: retrospective study on 5470 samples from 2001 to 2011. Mycopathologia. 2016;181(1-2):59-66. DOI: 10.1007/s11046-015-9953-7

4. Van Minnbruggen G, François IEJA, Cammue BPA, Thevissen K, Vroome V, Borgers M. *et al*. General overview on past, present and future antimycotics. The Open Mycol J. 2010;4(8):22-32.

5. Fernández-Torres B, Inza I, Guarro J. *In vitro* activities of the new antifungal drug erbeconazole and three other topical agents against 200 strains of dermatophytes. J Clin Microbiol. 2003;41:5209-11.

6. Mikhailov SN, Scotti L, Singla RK, Scotti T. Perspectives in medicinal chemistry. Curr Top Med Chem. 2016;16:2725-26.

7. Do TKT, Hadji-Minaglou F, Antoniotti S, Fernandez X. Authenticity of essential oils. Trends Analyt Chem. 2015;66:146-57.

8. Oliveira WA, Pereira FO, Luna CGDG, Lima IO, Wanderley PA, de Lima RB. *et al*. Antifungal activity of *Cymbopogon winterianus* Jowitt ex Bor against *Candida albicans*. Braz J Microbiol. 2011;42(2):433-41.

9. Freire MM, Jham GN, Dhingra OD, Jardim CM, Barcelos, RC, Valente VMM. Composition, antifungal activity and main fungitoxic components of the essential oil of *Mentha piperita* L. J Food Saf. 2012;32(1):29-36.

10. Kim E, Park IK. Fumigant antifungal activity of Myrtaceae essential oils and constituents from *Leptospermum petersonii* against three *Aspergillus* species. Molecules. 2012;17(9):10459-69.

11. Semmler M, Abdel-Ghaffar F, Schmidt J, Mehlhorn H. Evaluation of biological and chemical insect repellents and their potential adverse effects. Parasitol Res. 2014;113(1):185-88.

12. Pereira FO, Mendes JM, Lima IO, Mota KS, Oliveira WA, Lima EO. Antifungal activity of geraniol and citronellol, two monoterpenes alcohols, against *Trichophyton rubrum* involves inhibition of ergosterol biosynthesis. Pharm Biol. 2014;53(2):228-34. DOI: 10.3109/13880209.2014.913299

13. CLSI. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard M38-A. Pennsylvania: Clinical and Laboratory Standards Institute; 2008.

14. Santos DA, Hamdan JS. Evaluation of broth microdilution antifungal susceptibility testing conditions for *Trichophyton rubrum*. J Clin Microbiol. 2005;43(4):1917-20.

15. Hryncewicz- Gwozdza, Kalinowska K, Plomer-Niezgoda E, Bielecki JE, Jagielski T. Increase in resistance to fluconazole and itraconazole in *Trichophyton rubrum* clinical isolates by sequential passages in vitro under drug pressure. Mycopathologia. 2013;176(1-2):49-55.

16. Ghannoum M, Isham N, Verma A, Plaum S, Fleischer AJR, Hardas B. *In vitro* antifungal activity of naftifine hydrochloride against dermatophytes. Antimicrob Agents Chemother. 2013;57(9):4369-72. DOI: 10.1128/AAC.01084-13

17. Ghelardi E, Celandroni AF, Gueye SA, Salvetti S, Senesi S, Bulgheroni A. *et al*. Potential of ergosterol synthesis inhibitors to cause resistance or cross-resistance in *Trichophyton rubrum*. Antimicrob Agents Chemother. 2014;58(5):2825-29. DOI: 10.1128/AAC.02382-13

18. Osborne CS, Hofbauer B, Favre B, Ryder NS. *In vitro* analysis of the ability of *Trichophyton rubrum* to become resistant to terbinafine. Antimicrob Agents Chemother. 2003;47(11):3634-36.

19. Martins MP, Franceschini ACC, Jacob TR, Rossi A, Martinez-Rossi NM. Compensatory expression of multidrug-resistance genes encoding ABC transporters in dermatophytes. J Med Microbiol. 2016;65(7):605-10. DOI: 10.1099/jmm.0.000268

20. Santos AD, Araújo RAC, Santos AD, Kohler LM. Molecular typing and antifungal susceptibility of *Trichophyton rubrum* isolates from patients with onychomycosis pre- and post-treatment. Int J Antimicrob. Agents. 2007;29(5):563-69.

21. Borst A, Raimer MT, Warnock DW, Morrison CJ, Arthington-Skaggs BA. Rapid acquisition of stable azole resistance by *Candida glabrata* isolates obtained before the clinical introduction of fluconazole. Antimicrob Agents Chemother. 2005;49(2):783-87.

22. Pippi B, Lana AJ, Moraes RC, Güez CM, Machado M, de Oliveira LF. *et al*. *In vitro* evaluation of the acquisition of resistance, antifungal activity and synergism of Brazilian red propolis with antifungal drugs on *Candida* spp. J App Microbiol. 2015;118(4):839-50. DOI: 10.1111/jam.12746

23. Ram Y, Hadany L. Stress-induced mutagenesis and complex adaptation. Proc Biol Sci 2014;281(1792): 20141025. DOI:10.1098/rspb.2014.1025

24. Shor E, Perlin DS. Coping with stress and the emergence of multidrug resistance in fungi. PLoS Pathog. 2015;11(3):e1004668. DOI: 10.1371/journal.ppat.1004668

Conflict of Interest

The authors declare that they have no conflict of interest.

Author contributions

Gustavo Nunes Cardoso: Conceptualization; Data Curation; Writing – Original Draft.

Kaltz Victor Souza Silva: Conceptualization; Data Curation; Formal analysis.

Maria Islaine de Oliveira Lima: Conceptualization; Data Curation; Formal analysis.

Juliana Moura Mendes Arrua: Supervision; Writing – Review & Editing.

Fillipe de Oliveira Pereira: Conceptualization; Project administration; Writing – Review & Editing.