




# CYP51 Parologue Structure Is Associated with Intrinsic Azole Resistance in Fungi

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**ABSTRACT** Azoles are the most commonly used clinical antifungal therapy and also play an important role in control of plant pathogens. Intrinsic resistance to the azole class of fungicides, which target lanosterol demethylase (CYP51), is observed in many fungal species; however, the mechanisms underpinning this phenomenon are unknown. In this study, 5 azole-resistant *Penicillium* isolates from patients attending the UK National Aspergillois Centre that could not be morphologically identified to species level were analyzed by genome sequencing. The genomes and CYP51 parologue structure from these isolates were compared with those of 46 representative fungal isolates to identify to species level and examine possible mechanisms of drug resistance. Analysis of CYP51 paralogues showed that azole-resistant isolates from this study ( $n = 2$ ) and from public databases ( $n = 6$ ) contained a new CYP51 parologue, CYP51D, which was associated with azole resistance in 6/8 cases and never occurred in azole-sensitive species (46/46 tested). Furthermore, one isolate from this study and an azole-resistant *Aspergillus fumigatus* isolate were shown to encode a CYP51A parologue, CYP51A2. Introduction of CYP51A2 to the closely related but azole-sensitive *Aspergillus fumigatus* resulted in azole resistance. The identification of novel CYP51A and CYP51D paralogues in resistant fungi and the observation that resistance to azoles can be conferred by introducing a CYP51A parologue from a resistant species into an azole-sensitive species are a potentially important new azole resistance mechanism.

**IMPORTANCE** Azole antifungals are the main treatment for fungal disease in humans. Many species are intrinsically resistant to azoles—in other words all members of the species are resistant without prior exposure—and we do not understand why. In this study, we serendipitously discovered that many intrinsically resistant species have alternative or extra copies of the azole target gene, CYP51. Transfer of one of these genes from a resistant species to a sensitive one resulted in drug resistance, showing that the extra copies of CYP51 can confer drug resistance. Understanding how clinically important species are resistant to therapy allows us to predict whether a species could be resistant from genome sequence.

**KEYWORDS** *Aspergillus*, CYP51, *Penicillium*, antifungal agents, antifungal resistance, cryptic, paralogy

Fungi are a major cause of morbidity and mortality in humans and economically cause damaging crop loss in agriculture. One of the main methods to reduce fungal burden is treatment with azole antifungals which act by inhibiting CYP51 enzyme activity in the sterol biosynthesis pathway. In recent years resistance to azoles has become commonly reported and is an increasing concern in clinical and agricultural practice (1–3). Two main forms of resistance to azoles are observed. The first involves acquisition of mutations in the fungal genome which may include the target CYP51 enzyme, efflux pumps (4), or transcription factors regulating their function (5), and the second involves intrinsic resistance or insensitivity to the drug observed as a characteristic of certain species. For example, many filamentous

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fungi are intrinsically resistant to fluconazole due to the T301I substitution in the CYP51A protein (6, 7). This second form of azole insensitivity occurs in many species of clinical fungal pathogens and those of agricultural importance, and understanding this phenomenon is important in developing strategies to identify and counteract azole insensitivity.

The mechanism of intrinsic resistance or insensitivity in such species is not well understood. Most mold fungal species carry two CYP51 paralogues, CYP51A and -B. Despite this, the vast majority are azole sensitive. In the intrinsically resistant *Fusarium graminearum*, there are 3 CYP51 paralogues; however, the CYP51C paralogue appears to play no role in ergosterol biosynthesis or azole resistance (8–10). The rationale of the study was to use whole-genome sequencing to precisely define the species and to examine whether CYP51 duplication or paralogy could form the basis for an azole resistance mechanism in these fungi.

**CYP51 paralogue distribution correlates with azole resistance.** CYP51 paralogues were identified in the 5 test genomes (Table 1; see also Table S1 in the supplemental material). Surprisingly, the expected dual paralogue CYP51A-CYP51B pattern observed in previously studied *Aspergillus* and *Penicillium* genomes was observed only in the azole-sensitive *Penicillium glabrum* isolate. The azole-resistant *Penicillium olsonii* isolate (Pen spp. 18092) had 2 CYP51A (CYP51A1 and CYP51A2) and one CYP51B paralogues (Fig. 1, Table 1), and the 2 azole-resistant *Talaromyces* species (*T. diversus* and *T. radicus*) had no CYP51A paralogue but instead one CYP51B paralogue and a previously undescribed CYP51 paralogue more similar to CYP51B than to the *Fusarium* CYP51C, which we have termed CYP51D. Finally, the azole-resistant *Penicillium corylophilum* isolate contained the expected CYP51A and B paralogues but also a novel partial CYP51A gene (CYP51A-wtf [with truncated form]) corresponding to position 275 to C terminus of AFCYP51A). Promoters were checked for the presence of tandem repeats, but none were found.

The strains with both CYP51B and -D paralogues (CYP51B-D) were azole resistant, and this suggested that a more general analysis of CYP51 paralogues in other sequenced fungal strains might be fruitful in uncovering potential mechanisms of drug resistance. Representative genomes were selected with a focus on known clinical intrinsically resistant species (Table 1). Paralogues were assigned in each genome as described and checked for correct 1-to-1 orthology by reverse BLAST. The CYP51B-D paralogue structure was observed in a subgroup of *Talaromyces* species (*T. stipitatus* ATCC 10500, *T. borbonicus* CBS 141340, *T. aculeatus* ATCC 10409, *T. proteolyticus* PMI\_201, and *T. amestolkiae* strain CIB) also noted to be intrinsically azole resistant. A CYP51A, A2, and B paralogue pattern was observed in the azole-resistant species *Aspergillus fumigatiaffinis*. We note that the intrinsically resistant *Aspergillus lentulus* has a normal CYP51A-B paralogy pattern. In comparison all *Fusarium* spp. analyzed had the CYP51A-B-C paralogue structure previously reported.

The nature and mechanisms underpinning intrinsic azole resistance in molds are unknown. Here, we show the existence of a new CYP51 paralogue, CYP51D, which predominantly occurs in genomes of intrinsically azole-resistant Eurotiomycetes. Although we did not observe a perfect correlation between paralogue pattern and intrinsic resistance, this would be expected as many other genomically encoded factors are known to contribute to azole tolerance. The observed correlation was striking with 5/8 species carrying the CYP51B-D paralogue structure being azole resistant, 2/8 displaying intermediate sensitivity, and 1/8 having unknown sensitivity. All 46/46 genome-sequenced azole-sensitive isolates examined in this study had the CYP51A-B paralogue structure; however, 2 intrinsically resistant species, *Lomentospora prolificans* and *A. lentulus*, also had a CYP51A-B paralogue structure. Intrinsically resistant *Pseudallescheria* species carried a single CYP51B paralogue.

**Transfer of the CYP51A2 paralogue from *A. fumigatiaffinis* to *Aspergillus fumigatus* confers itraconazole resistance.** Two species, *A. fumigatiaffinis* and *P. olsonii*, had a CYP51A1-A2-B paralogue structure with one species known to be intrinsically resistant and the other noted as resistant in this study. In order to determine the relation between CYP51A2 and azole resistance, we transformed the *A. fumigatiaffinis* CYP51A2 into *A. fumigatus* A1160p+ (Fig. 2a, Fig. S1, and Table S2). While no apparent defects in radial growth or morphology were observed in the transformant (Fig. 2b), A1160p+CYP51A2 was resistant to

**TABLE 1** CYP51 paralogue distribution in fungal species<sup>a</sup>

Strain	CYP51A1	CYP51A2	CYP51B	CYP51D	CYP51wtf	Azole res.
<i>T. atrovirens</i> IBT 11181	—	—	+	—	—	?
<i>T. pinophilus</i> AR155	—	—	+	+	—	?
<i>T. amestolkiae</i> strain CIB	—	—	+	+	—	I
<i>T. borbonicus</i> CBS 141340	—	—	+	+	—	I
<i>A. lentulus</i> CBS 117885	+	—	+	—	—	R
<i>P. corylophilum</i> 23421	+	—	+	—	+	R
Pen spp. 18092 ( <i>P. olsonii</i> )	+	+	+	—	—	R
Pen spp. 26444 ( <i>T. diversus</i> )	—	—	+	+	—	R
Pen spp. 30722 ( <i>T. radicus</i> )	—	—	+	+	—	R
<i>T. aculeatus</i> ATCC 10409	—	—	+	+	—	R
<i>T. proteolyticus</i> PML_201	—	—	+	+	—	R
<i>T. stipitatus</i> ATCC 10500	—	—	+	+	—	R
<i>Lomentospora prolificans</i>	+	—	+	—	—	R
<i>Pseudallescheria boydii</i>	—	—	+	—	—	R
<i>Pseudallescheria apiospermum</i>	—	—	+	—	—	R
<i>Pseudallescheria dehoogii</i>	—	—	+	—	—	R
<i>Pseudallescheria aurantiacum</i>	—	—	+	—	—	R
<i>A. fumigatiaffinis</i>	+	+	+	—	—	R
<i>A. fumisynnematus</i>	+	—	+	—	—	S
<i>A. fischeri</i> NRRL 181	+	—	+	—	—	S
<i>A. fumigatus</i> A1163	+	—	+	—	—	S
<i>A. fumigatus</i> AF293	+	—	+	—	—	S
<i>A. fumigatus</i> Z5	+	—	+	—	—	S
<i>Neosartorya hirsutiae</i> CBS 294.93	+	—	+	—	—	S
<i>P. expansum</i> MD8	+	—	+	—	—	S
<i>P. antarcticum</i>	+	—	+	—	—	S
<i>P. arizonense</i>	+	—	+	—	—	S
<i>P. atramentosum</i> RS17	+	—	+	—	—	S
<i>P. biforme</i> FM169	+	—	+	—	—	S
<i>P. brasilianum</i> ZJ-7	+	—	+	—	—	S
<i>P. brasilianum</i>	+	—	+	—	—	S
<i>P. brasilianum</i> LaBioMMi136	+	—	+	—	—	S
<i>P. camemberti</i> FM013	+	—	+	—	—	S
<i>P. capsulatum</i> ATCC 48735	+	—	+	—	—	S
<i>P. capsulatum</i> LiaoWQ-2-011	+	—	+	—	—	S
<i>P. carnescens</i> ATCC 10419	+	—	+	—	—	S
<i>P. carneum</i> LCP05634	+	—	+	—	—	S
<i>P. chrysogenum</i> 1B	+	—	+	—	—	S
<i>P. chrysogenum</i> HK F42	+	—	+	—	—	S
<i>P. chrysogenum</i> KF25	+	—	+	—	—	S
<i>P. chrysogenum</i> NCPC 10086	+	—	+	—	—	S
<i>P. chrysogenum</i> P2niaD18	+	—	+	—	—	S
<i>P. citrinum</i>	+	—	+	—	—	S
<i>P. citrinum</i> DSM 1997	+	—	+	—	—	S
<i>P. coprophilum</i> IBT 31321	+	—	+	—	—	S
<i>P. decumbens</i> IBT 11843	—	—	+	—	—	S
<i>P. digitatum</i> Pd1	+	—	+	—	—	S
<i>P. digitatum</i> Pd1 ZJU	+	—	+	—	—	S
<i>P. digitatum</i> PDC 102	+	—	+	—	—	S
<i>P. digitatum</i> PHI 26	+	—	+	—	—	S
<i>P. expansum</i> d1	+	—	+	—	—	S
<i>P. expansum</i> NRRL 62431	+	—	+	—	—	S
<i>P. expansum</i> R19	+	—	+	—	—	S
<i>P. expansum</i> T 01	+	—	+	—	—	S
<i>P. expansum</i> YT02	—	—	+	—	—	S
<i>P. glabrum</i> 23851	+	—	+	—	—	S
<i>P. griseofulvum</i> PG3	+	—	+	—	—	S
<i>P. italicum</i> PHI1	+	—	+	—	—	S
<i>P. polonicum</i> IBT 4502	+	—	+	—	—	S
<i>P. raistrickii</i> ATCC 10490	+	—	+	—	—	S
<i>P. solitum</i> IBT 29525	+	—	+	—	—	S
<i>P. swiecickii</i> 182 6C1	+	—	+	—	—	S

(Continued on next page)

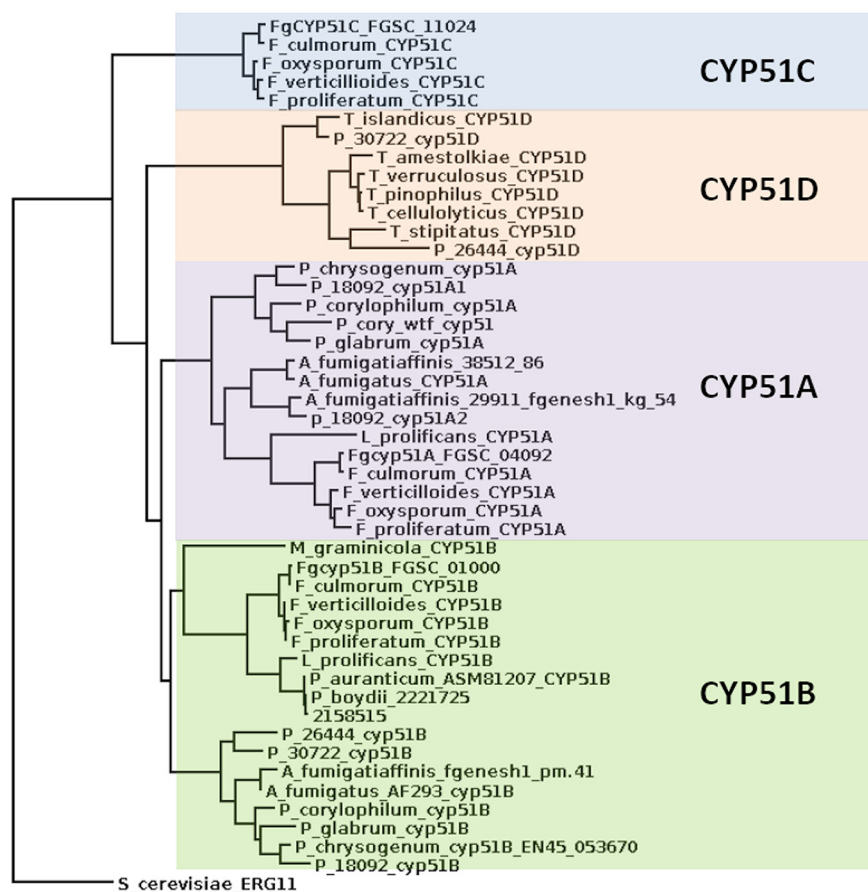
**TABLE 1** (Continued)

Strain	CYP51A1	CYP51A2	CYP51B	CYP51D	CYP51wtf	Azole res.
<i>P. thymicola</i> DAOMC 180753	+	–	+	–	–	S
<i>P. expansum</i> CMP1	+	–	+	–	–	S
<i>T. marneffei</i> ATCC 18224	–	–	+	–	–	S
<i>T. purpureogenus</i> MYA-38	+	–	+	–	–	S

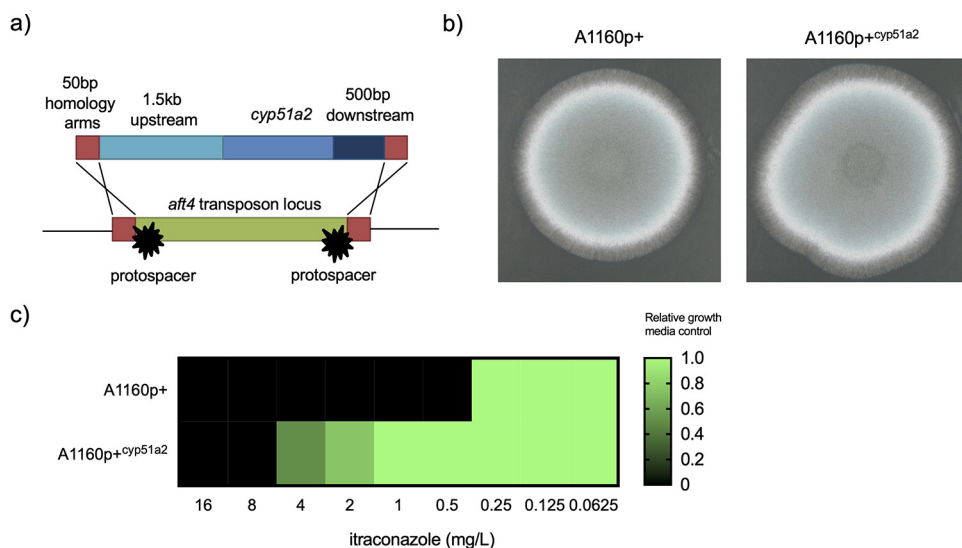
<sup>a</sup>Paralogues were assigned in each species according to their position in the phylogeny. Resistance for the species was assigned for MICs in the current study or previous literature. *Penicillium* isolates identified in this study are shaded in gray and are single isolates. Symbols and abbreviations: +, paralogue present in the genome; –, paralogue absent in the genome; res., resistance; R, all reported isolates from the species or the average MIC for the species shows azole resistance (>MIC breakpoint for either itraconazole, voriconazole, or posaconazole); I, intermediate resistance observed in the species;?, azole resistance not tested in the species; S, no resistance observed in the species.

itraconazole (8 mg/liter) compared to the parental isolate (0.5 mg/liter) (Fig. 2c). MICs observed for this *A. fumigatus* strain are comparable to the MIC determined for *A. fumigatiaffinis* isolates (11). The presence of two paralogous, nonidentical CYP51A genes could potentially explain the observed azole resistance in *A. fumigatiaffinis*.

It should be stressed that the current study shows only a correlation between the presence of CYP51D and azole resistance ( $P < 0.0001$  assuming random selection of genomes) but that no causal relationship has been tested for CYP51D. The *Fusarium* CYP51C paralogue does not appear to play a role in either azole resistance or ergosterol biosynthesis; however, CYP51D is unrelated to CYP51C and groups more closely with CYP51B (Fig. 1). This may support a role for CYP51D in ergosterol biosynthesis, but considerable further work is required to confirm this hypothesis. Additionally, the analysis presented here could be limited by the availability of genome sequences which may be biased toward “interesting” genomes displaying phenotypes such as drug insensitivity. Perusal of the available genomes does not



**FIG 1** Illustrative phylogeny of CYP51 paralogue genes in this study. Typical CYP51 paralogue genes were selected. Complete protein sequences were aligned using MUSCLE, and phylogenies were calculated using FastTree with -gamma -spr 4 -mlacc 2 -slow parameters.



**FIG 2** *A. fumigatiaffinis cyp51a2* knock-in results in resistance. (a) Overview of replacing the nonfunctional *aft4* transposon locus with the *A. fumigatiaffinis cyp51a2* gene including 1.5 kb upstream and 500 bp downstream. Microhomology arms of 50 bp are included to allow CRISPR-Cas9-mediated transformation. (b) A phenotypic test on *Aspergillus* complete medium, grown for 48 h at 37°C. (c) MIC assay for itraconazole for both the parental isolate (A1160p+) and A1160p+cyp51a2. Growth was normalized to nondrug control (1% dimethyl sulfoxide [DMSO]).

support this, with most arising from projects aimed at use of certain species in food or antibiotic production or primarily as pathogens with no focus on drug sensitivity. There is little depth in the literature describing azole resistance in many of the species included in this paper, and it would be desirable to perform a survey of multiple representatives of each species to properly assess MICs for several azoles to support these findings. Finally, our focus on CYP51D does not preclude the possibility that lack of the CYP51A paralogue in CYP51B-D paralogue genomes may also affect azole resistance as this is potentially the primary target of azoles. Isolates carrying the CYP51B-D paralogues grew in a normal manner and exhibited abundant sporulation, so presumably they are able to synthesize adequate sterol membrane components.

The observation that transfer of a CYP51A2 paralogue from a resistant species into a sensitive species results in azole resistance suggests that intrinsic azole resistance can arise from CYP51 paralogue structure, and this observation, together with an accurate understanding of paralogue number and type, will provide an important guide to understanding intrinsic azole insensitivity or tolerance in fungal species. We note that the CYP51A2 allele from *A. fumigatiaffinis* does not have altered amino acids at positions previously identified as being important for resistance in the *A. fumigatus* CYP51A gene, such as L98, G54, or M220. This may mean that higher protein levels resulting from expression of 2 gene copies rather than failure of drug to bind protein could explain the altered azole sensitivity. Further confirmation of such observations, for example, by deletion of paralogues in intrinsically resistant species, awaits development of appropriate transformation methodology in these species.

**Experimental procedures.** Five *Penicillium* species isolates were obtained from the NHS Mycology Reference Centre, Manchester, United Kingdom. The isolates had been tentatively identified as *Penicillium* spp. by morphology, and azole resistance was determined by MICs for itraconazole, voriconazole, and posaconazole using EUCAST procedures (12).

**(i) Genome sequencing.** DNA was prepared as previously described for *Aspergillus fumigatus* (4) and sequenced with a paired-end protocol using an Illumina HiSeq2000. Genomes were assembled using Velvet (13) with a 31-bp kmer input and queried using BLASTN and TBLASTN (14) and a set of *Aspergillus fumigatus*, *Fusarium graminearum*, and *Penicillium chrysogenum* CYP51 query protein sequences (Fgcyp51A; FGSC\_04092, Fgcyp51B; FGSC\_01000, FgCYP51C; FGSC\_11024, *P. chrysogenum* CYP51A; EN45\_094440, *P. chrysogenum* CYP51B; EN45\_053670). Orthology and paralogy were confirmed by BLAST



back to the original reference genomes. Genomes for comparison were obtained from NCBI (accessed May 2020) and JGI databases (15, 16), and coordinates for each CYP51 paralogue were compared (Table S1). Sequences for  $\beta$ -tubulin and the RNA polymerase subunits RPB1 and  $-2$  were also extracted using TBLASTN with query sequences derived from *P. chrysogenum* and used for species identification. BLAST analysis was handled using bash scripted pipelines.

Predicted CYP51 protein sequences were aligned using MUSCLE, manually checked and corrected, and then trimmed to remove unconserved N-terminal residues before phylogenetic tree construction (Fig. 1). Data on azole sensitivity and phylogenies for *Penicillium* and *Talaromyces* were obtained from previous publications (17–21).

Genome sequencing and assembly resulted in  $N_{50}$  of  $>80$  kb and coverage of  $>75\times$  for all genomes. Genomes and genome assemblies from public databases all also showed similar or better assembly profiles.

**(ii) Genome-based identification of isolates.** The 5 *Penicillium* isolates were identified using  $\beta$ -tubulin, RPB1, and RPB2 species-level identification markers as previously defined (21). Protein sequences derived from the isolate genome sequence assembly compared to publicly available genome sequences confirmed the identity of strain 23421 as *P. corylophilum* (100% protein identity [ID] to genomic *P. corylophilum*  $\beta$ -tubulin, RPB1 and RPB2 genes) and 23851 as *P. glabrum* (99, 99, and 100% ID, respectively). *Penicillium* species 30722 was identified as *Talaromyces radicus* (99, 100, and 100% ID, respectively), *Penicillium* species 26444 was identified as *Talaromyces diversus* (99, 100, and 100% ID, respectively), and *Penicillium* species 18092 was identified as *P. olsonii* (99, 100, and 100% ID, respectively).

**(iii) CYP51A2 transfer into *A. fumigatus*.** The CYP51A2 gene was amplified from *A. fumigatiaffinis* genomic DNA using primers cyp51a2\_Fw and cyp51a2\_Rv (Table S2) including 1.5 kb upstream and 500 bp downstream of the predicted open reading frame (ORF), followed by gel purification (NucleoSpin; Macherey-Nagel). CYP51A2 was transformed into a genomic “safe haven” in *A. fumigatus* A1160p+ using selection-free CRISPR-Cas9-mediated transformation with crRNA atf4\_TIRm\_up and atf4\_TIRm\_down (22, 23). Transformants were purified twice on Sabouraud agar and PCR verified using cyp51a2\_chk\_fw and atf4\_screening\_rv. A phenotypic assay was performed by spotting 1,000 spores in  $5\ \mu\text{l}$  onto *Aspergillus* complete medium (ACM) incubated for 48 h at  $37^\circ\text{C}$ . MIC assays for itraconazole were performed according to EUCAST methodology (12).

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**FIG S1**, TIF file, 0.4 MB.

**TABLE S1**, XLSX file, 0.02 MB.

**TABLE S2**, XLSX file, 0.01 MB.

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We have no conflicts of interest to report.

N.V.R., P.B., M.B., and M.R. conceived and designed the study, N.V.R. and M.B. performed the lab work, and P.B. wrote the manuscript with revisions by N.V.R., M.B., and M.R.

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