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The Evaluation of the Combination of Caspofungin with Voriconazole and Amphotericin B against Clinical *Candida krusei* Isolates by Etest and Disk Diffusion Methods

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Authors' contributions

This work was carried out in collaboration between all authors. Authors YO and NK designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors YO and AK managed the analyses of the study. Author FA managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Background: *C. krusei* is an opportunistic fungal pathogen that known of its intrinsic fluconazole resistance and its frequency is increasing especially among hematology patients. The increase in the frequency of high mortality fungal infections have accelerated the efforts of new drug development with broad spectrum, low toxicity and the studies including their combination. However, there is no standardized method to evaluate the activity of drug combinations.
Aims: To evaluate the activity of caspofungin (CAS) with voriconazole (VOR) and amphotericin B (AMB) alone and in combination and the utility of Etest and disk diffusion methods for antifungal combinations.
Methodology: The minimum inhibitory concentrations of VOR, CAS and AMB against 30 clinical *C. krusei* isolates were determined by using Etest, disk diffusion and reference broth microdilution methods. Combinations of CAS with VOR and CAS with AMB were evaluated using disk diffusion (three different ways) and Etest (two different ways) methods.

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Results: All isolates tested were susceptible to VOR and CAS in vitro by all three methods. Categorical agreements of Etest and disk diffusion methods with reference microdilutiontest were 100% for CAS and VOR (for each method), 86.7% and 50% for AMB, respectively. In the all ways of both combination methods, we did not observe distinctly antagonistic or synergic interaction.
Conclusion: Etest and disk diffusion could be easy, convenient, and nontime-consuming alternative methods to evaluate the antifungal combinations. The combinations of CAS with VOR and AMB exhibited promising results because of an apparent antagonistic interaction was not detected in this study.

Keywords: Antifungal combination; Etest; Disk diffusion; Candida krusei.

ABBREVIATIONS

CAS: caspofungin; VOR: voriconazole; AMB: amphotericin B; S: susceptible; SDD: susceptible-dose dependent; R: resistant; BM: broth microdilution; DD: disk diffusion; MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration; CLSI: Clinical and Laboratory Standards Institute.

1. INTRODUCTION

More than 90% of invasive candidiasis are attributed to five *Candida* species; *C. albicans*, *C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei*. However, Hachem et al. reported that *C. glabrata* and *C. krusei* were the leading causes of candidemia in patients with hematological malignancy [1]. *C. krusei* is resistant to fluconazole intrinsically and there are several reports that decreased susceptibilities to amphotericin B and flucytosine were noted [2].

The increase of the frequency of opportunistic fungal infections with high mortality has accelerated the development efforts of new drugs with broad spectrum, low toxicity and the studies of their combination. The aims of combination therapy are to provide the more effective and broad spectrum of efficacy, generate the greater potency than either of the drugs used in monotherapy, improve the safety and tolerability by using lower dosage, reduce the number of resistant organisms. However, combined usage of drugs could reduce the antifungal efficacy, increase the toxic side effects and increase the cost of antifungal therapy. Therefore, the evaluation of drug interactions is important.

Although there is a great interest to studies including the use of antifungal combinations for treatment of invasive fungal infections, there is no standardized method evaluating the antifungal drug interactions. For this purpose, checkerboard dilution is the most common used method, but the time-kill method is less preferred because of labor-intensive and time-consuming. Also Etest method has been used to evaluate the drug interactions in several combination studies and it has been reported as a practical and reproducible method [3,4]. We aimed to evaluate the activity of combination of caspofungin (CAS) with voriconazole (VOR) and amphotericin B (AMB) against clinical isolates of *C. krusei* using Etest and disk diffusion methods.

2. MATERIALS AND METHODS

2.1 Isolates

A total of 30 clinical isolates of *C. krusei* from various specimens were included in this study. The identification of isolates was performed using conventional methods and commercially available tests such as CHROMagar Candida (BD Diagnostic, Sparks, MD) and API 20C (bioMérieux, Marcy l'Etoile, France). We also used *Candida parapsilosis* ATCC 22019 as a quality control isolate.

2.2 Antifungal Agents

CAS (Merck Research Laboratories, Rahway, USA), VOR (Pfizer, New York, USA) and AMB (Sigma, St Louis, MO, USA) were obtained from their manufacturers, CAS was dissolved in distilled water; VOR and AMB were dissolved in dimethyl sulfoxide. Stock solutions were prepared in RPMI 1640 medium (Sigma) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma), microplates with serial twofold dilutions ranging 0.03-16 µg/ml of each drug were prepared and stored at -70ºC until use. CAS, VOR and AMB Etest strips were purchased from manufacturer (AB Biodisk, Solna, Sweden).

2.3 Antifungal Susceptibility Testing

2.3.1 Broth microdilution method

The minimum inhibitory concentration (MIC)s of VOR, CAS and AMB for each isolate were determined by using Clinical and Laboratory Standards Institute (CLSI) document M27-A2 broth microdilution (BM) method [5]. RPMI 1640 medium buffered to pH 7.0 with MOPS was used for this purpose. All isolates were subcultured onto Sabouraud dextrose agar and the inoculum was prepared spectrophotometrically after 24-48 hours of incubation. A standard 0.5 McFarland fungal suspension was prepared with sterile 0.85% saline. It was diluted using RPMI 1640 broth medium to obtain a starting inoculum of $1\text{-}5x10^3$ cells per ml. Microtiter plates were inoculated and incubated at 35ºC and the MICs were read visually after 48h. Endpoints for CAS and VOR were defined as the lowest concentration of drug that resulted in a prominent (approximately 50%) reduction of visual growth compared with the growth control wells. MICs of AMB were defined as the lowest concentration of drug which resulted in total inhibition of visual growth [3]. In addition, minimal fungicidal concentration (MFC) values were obtained by plating 0.1 ml from clear MIC wells following 48h of incubation onto Sabouraud dextrose agar plates [6]. The MFC was the lowest drug concentration that resulted in ≤1 colony. All tests were carried out in duplicate.

2.3.2 Disk diffusion test

Whatman no 1 filter papers were used to prepare the antifungal disks. Disks of 6.3 mm in diameter sterilized in Pasteur oven were impregnated with CAS, VOR and AMB suspensions, resulting in final concentrations 5, 1 and 10 µg/disk, respectively [3,7]. Disk diffusion tests were performed as recommended by the CLSI for yeasts using Mueller-Hinton agar supplemented with 2% glucose and 0.5% methylene blue [8]. Standard 0.5 McFarland fungal suspensions were prepared and each plate was streaked with a sterile cotton swab in

three directions. All plates were allowed to dry for 15 minutes, then disks were applied onto the surface of each plate, after that the plates were incubated at 35ºC. Zone diameters were measured at the point in which there was a prominent reduction (80%) for CAS and VOR, complete inhibition for AMB in growth after both 24h and 48h [3,7]. All tests were carried out at least in duplicate.

2.3.3 Etest method

Testing was performed on RPMI 1640 agar plates buffered to pH 7.0 with MOPS as recommended by the manufacturer. Standardized 0.5 McFarland turbidity yeast suspension was spread to the entire agar surface in three directions. The Etest antifungal strips were placed onto inoculated the agar by using sterile forceps. Plates were incubated at 35ºC for 48h. MICs were read after 24 and 48 h of incubation and were defined as 80% inhibition for CAS and VOR, complete inhibition for AMB [9]. All tests were carried out in duplicate.

2.4 Evaluation of Antifungal Susceptibility

The interpretive breakpoint values for VOR disk tests were described as follows; zone diameters of ≥17 mm were susceptible (S), 14-16 mm were susceptible-dose dependent (SDD) and ≤13 mm were resistant (R); the corresponding MIC breakpoints were as follows; ≤1 µg/ml, 2 µg/ml and ≥4 µg/ml, respectively [10]. The interpretive criteria for CAS disk tests was considered as zone diameters of ≥11 mm were S and ≤10 mm were nonsusceptible; the corresponding MIC breakpoints were ≤2 µg/ml and >2 µg/ml, respectively [3,11]. The interpretive breakpoint for AMB disk test was defined as zone diameters ≤10 mm were R and MIC breakpoints ≤1 µg/ml were S, ≥2 µg/ml were R [12].

2.5 Antifungal Combination Studies

Combinations of CAS with VOR (CAS+VOR) and CAS with AMB (CAS+AMB) were evaluated using disk diffusion (three different ways) and Etest (two different ways) methods.

2.5.1 Disk Diffusion Method

Antifungal combinations were studied in three different ways simultaneously on Mueller-Hinton agar with glucose and methylene blue; firstly, the distance between disks was adjusted as the sum of the radii obtained when each drug was tested alone; second, disks were placed with a distance to be half of the radius sum of drugs and thirdly, one disk included two antifungal agents together. Plates were evaluated in terms of the presence of bridging, expansion or disruption in the junction of the inhibition zones after 24 h incubation at 35ºC. The combination of two drugs combined in one disk was compared with that achieved by the most active agent [13]. All tests were carried out at least in duplicate.

2.5.2 Etest method

Combination studies were performed in two different ways using Etest. First; CAS Etest strip was placed on agar and removed after 1h. VOR or AMB Etest strips were placed on agar over demarcation left from CAS strip. MICs were read after 48h incubation at 35ºC. Synergy was defined as a decrease of ≥3 dilution, indifference was a change of <3 dilution and antagonism was defined as an increase of ≥3 dilution in the combination MIC compared with resultant MIC [4]. In the second Etest combination way, strips were placed on the agar

plates in a cross formation with a 90º angle at the previously described MIC levels alone [14]. Results were evaluated in terms of expansion or narrowing in region between strips after 48h incubation at 35ºC. All tests were carried out in duplicate.

3. RESULTS AND DISCUSSION

Antifungal susceptibility test results of all isolates were summarized in Table 1. All of the *C. krusei* isolates tested were evaluated as in vitro susceptible to VOR and CAS by the used three methods. AMB gave the highest MIC values in both microdilution and Etest methods. Etest MIC values tended to be lower than BM MICs in general. The widest susceptibility zone diameters by disk diffusion method were observed with CAS. Diversity between 24 and 48 h incubation results was not detected for CAS and VOR; whereas 1-3 mm reduction was observed in AMB zone diameters for 28 isolates after 48h. Categorical agreements of Etest and disk diffusion with reference microdilution test were good for CAS and VOR but lower for AMB. CAS and AMB MFCs were lower than that of VOR; CAS and AMB MFCs were ≤3 dilutions of the MICs but VOR MFCs were ≥3 dilutions of the MICs.

We compared our susceptibility results from CLSI BM with those recently reported in literature for *C. krusei* and VOR, CAS, AMB (Table 2). We observed that our results are in agreement with those reported previously.

When two drugs were combined in one disk; zone diameters of the combined disks did not change markedly compared with the most active drug alone; it was observed to be narrower (1-6 mm) for 17 isolates, expansion (1-3 mm) for 8 isolates and no discrepancy for 5 isolates in the CAS+VOR combinations; it was found to be narrower (1-5 mm) for 10 isolates, expansion (1-3 mm) for 6 isolates and no discrepancy for 14 isolates in the CAS+AMB combinations (Fig. 1). For the other two DD combination ways, any interaction between two antifungals was not observed independently from distance between disks (Fig. 1).

Table 1. Antifungal susceptibility results of 30 clinical *C. krusei* isolates

Antifungals	Method[a]	MIC Range[b]	MIC_{50} µg/ml	MIC_{90} µg/ml	Geometric Mean	% Categorical agreement	% Agreement within ±2 dilution
Caspofungin	BM	0.25-1.0	0.5	1.0	0.56		
	MFC	0.5-8	1	2	1.61		
	ET	0.047-0.5	0.25	0.38	0.28	100	83.3
	DD	25-30	NA	NA	27.6	100	NA
Voriconazole	BM	0.25-0.5	0.5	0.5	0.41		
	MFC	1-16	8	16	6.96		
	ET	0.094-0.5	0.19	0.38	0.24	100	100
	DD	18-30	NA	NA	23	100	NA
Amphotericin B	BM	1.0-2.0	1.0	2.0	1.5		
	MFC	2-8	2	8	3.13		
	ET	0.5-2.0	1.5	2.0	1.39	86.7	100
	DD	18-24	NA	NA	20.13	50	NA

[a]*BM, broth microdilution; MFC, minimal fungicidal concentration; ET, Etest; DD, disk diffusion*
[b]*µg/ml for MD and ET; mm for DD.NA, not applicable.*

In the first Etest combination way; lower MIC values were detected when compared with the results from each drug alone but interaction was not evaluated as synergy because the results remained within ±3 dilution for VOR+CAS combinations. Conversely, CAS+AMB

combination MIC values were higher than that of each drug alone and antagonism was defined for the two isolates. However, this antagonistic interaction was not observed in the other combination tests in this study. In the second Etest combination way; any alteration was not detected in the region between strips for both combinations (Fig. 2).

Table 2. A comparison of our in vitro susceptibility results with the previous studies on *C. krusei* published in the last ten years

Drugs and References	No of isolates	MIC Range (µg/ml)	MIC 50 (µg/ml)	MIC 90 (µg/ml)	Methods*
Voriconazole					
Pfaller et al. [10]	243	0.06-1.0	0.25	0.5	CLSI
Borghi et al. [15]	15	0.12-1.0	0.25	1.0	CLSI
Lockhard et al. [16]	32	0.015-1.0	0.25	0.5	CLSI
Peman et al. [17]	27	0.03-2.0	0.25	0.25	Sensititre Yeast One
Arendrup et al. [18]	52	0.125-4.0	0.25	0.5	EUCAST
Fleck et al. [19]	19	0.06-1.0	0.25	1.0	CLSI
Maxwell et al. [20]	118	0.06-2.0	0.25	0.5	CLSI
Present study	30	0.25-0.5	0.5	0.5	CLSI
Caspofungin					
Pfaller et al. [2]	300	0.03-2.0	0.12	0.25	CLSI
Canton et al. [3]	20	0.5-2.0	1.0	2.0	CLSI
Lockhard et al. [16]	32	0.047-2.0	0.38	0.5	Etest
Peman et al. [17]	27	0.03-8.0	0.25	0.5	Sensititre Yeast One
Arendrup et al. [18]	25	0.25-0.5	0.5	0.5	Etest
Fleck et al. [19]	19	0.5-10	1.0	1.0	CLSI
Ranque et al. [21]	10	0.25-1.0	0.5	0.5	CLSI
Present study	30	0.25-1.0	0.5	1.0	CLSI
Amphotericin B					
Pfaller et al. [2]	304	0.03-16	1.0	4.0	Etest
Borghi et al. [15]	15	0.5-2.0	1.0	2.0	CLSI
Lockhard et al. [16]	32	0.25-6.0	2.0	4.0	Etest
Peman et al. [17]	27	0.12-1.0	0.5	1.0	Sensititre Yeast One
Arendrup et al. [18]	52	0.25-4.0	1.0	2.0	Etest
Fleck et al. [19]	19	0.5-2	1.0	1.0	CLSI
Ranque et al. [21]	10	0.5-1.0	1.0	1.0	CLSI
Kiraz& Oz [22]	74	0.03-2.0	0.75	2.0	Etest
Present study	30	1.0-2.0	1.0	2.0	CLSI

**The results read at 48h. for CLSI broth microdilution test were included in this table.*

Although standardized methods for in vitro antifungal susceptibility testing have been proposed by CLSI, commercially available agar diffusion techniques such as Etest and disk diffusion are widely used in routine clinical laboratories. The Etest is a reproducible, reliable and practical method and have good agreement values (≥89%) with the reference BM method [9,19,20]. Our agreement results (>83%) between Etest and reference BM test were similar to results of these studies. Although the agreement between Etest and BM can be different depending on the various variables such as species of the fungus, kind of the drug, and incubation time, it was in the acceptable ranges in general. The high MFC values of VOR were related with its fungistatic activity against *Candida* spp., whereas in relation to the fungicidal effect of CAS and AMB, their MFC values were close to the MICs.

Reference susceptibility testing is micro/macro broth dilution method proposed by CLSI. In addition, disk diffusion test has been standardized for fluconazole and voriconazole against yeasts [8]. It has several advantages such as lower cost, reduced labor and practicality, nontime-consuming and easy feasibility. In our study, zone diameters of all isolates tested were ≥25 mm for CAS, ≥18 mm for VOR and ≥18 mm for AMB by the disk diffusion method. In this case, all isolates were susceptible to CAS and VOR; none of the isolates were resistant to AMB. Pfaller et al. [10,23] have reported >94% and 99% categorical agreement between disk diffusion and reference method for VOR in their two different multicenter studies.

Studies including CAS and AMB disk diffusion testing were limited. Espinel-Ingroff et al. [7] have reported that categorical agreements were 97-100% for CAS, 96% for VOR and 100% for AMB. However, authors used different interpretive criteria than that in our study; S, ≥14 mm and R,≤13 mm for CAS zone diameters and S, ≥15 mm and R, ≤13 mm for AMBzone diameters. We detected 100% categorical agreement for CAS and VOR but it was very low for AMB. The standardization for antifungals other than fluconazole and VOR has not been constituted to detect the antifungal susceptibility by disk diffusion method. However, we think that, disk diffusion test can be used commonly in most routine clinical laboratory when it was also standardized for other antifungals.

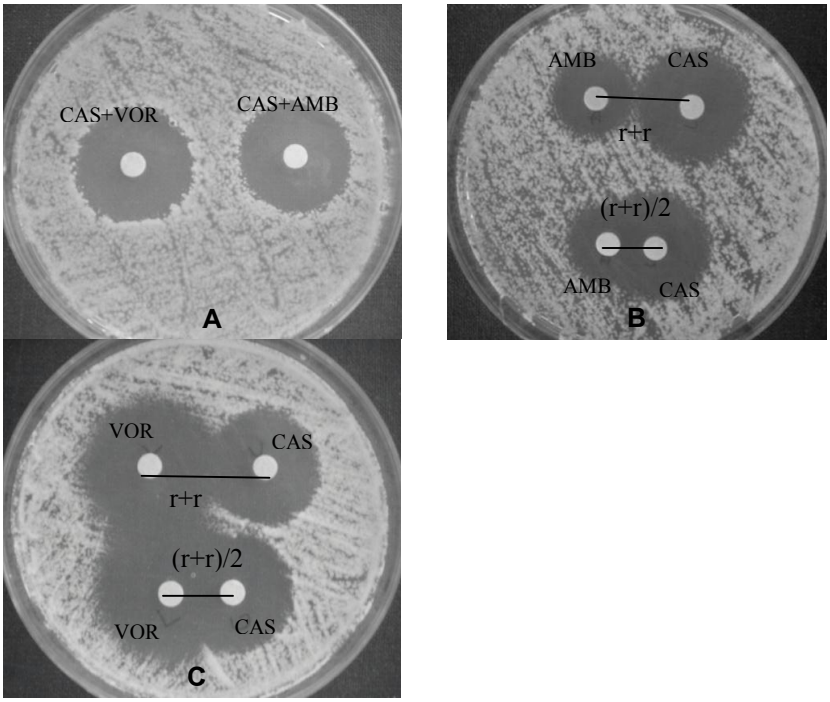

Fig. 1. Three different disk diffusion methods

A, One disk with two antifungal agents together; B and C, the distance between the disks is the sum of radii obtained when each drug was tested aloneand the distance between disks is half of the radius sum of drugs on Mueller-Hinton agar with glucose and methylene blue. CAS, caspofungin; VOR, voriconazole; AMB, amphotericin B; r, radius.

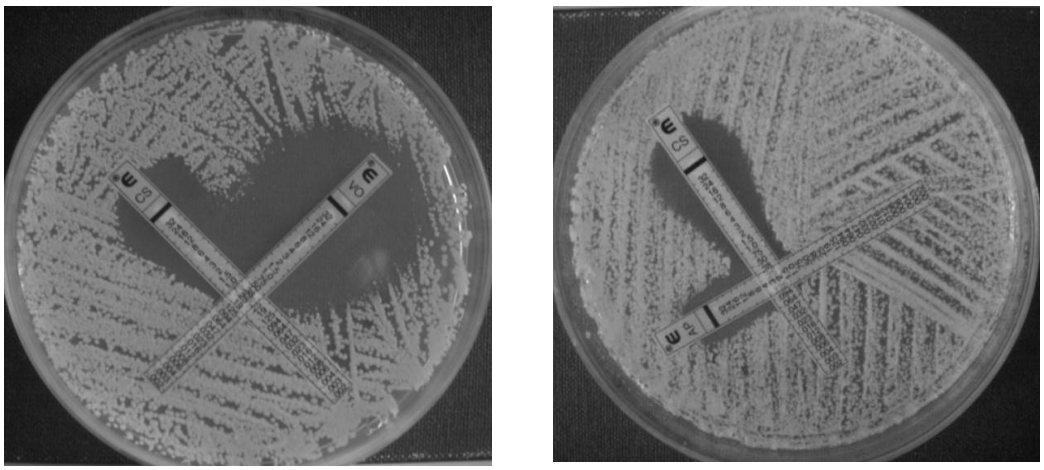

Fig. 2. Second Etest combination method

Strips were placed onto RPMI 1640 agar plates in a cross formation with a 90º angle at the previously described MIC levels alone. CS, caspofungin; VO, voriconazole; AP, amphotericin B.

The methods to detect the antifungal susceptibility have been developed by CLSI but the methods to evaluate the activity of antifungal combinations have not been standardized yet. The most common used test is checkerboard microdilution method for this purpose; however time-kill test is more useful because it provides knowledge about dynamics of combination. It should be noted that, time-kill test is laborious and time-consuming.

For the purpose of determining the activity of antifungal combinations, number of studies evaluating the Etest and DD methods is very limited. Lewis et al. [4] found a good agreement between the Etest and time-kill methods, but they reported that, checkerboard dilution method may not reliably detect the activity of combination especially for AMB in RPMI medium. Ernst et al. [24] detected that Etest was more concordant with time-kill method than checkerboard dilution method. In our previous study, we found 66-86%agreement between Etest and time-kill methods in combinations of CAS and azoles against *C. glabrata* [13].

Barchiesi et al. [25] evaluated the combination of CAS and AMB against *C. parapsilosis* isolates using checkerboard, disk diffusion and *time-kill* methods. They detected 100% indifferent interactions by the checkerboard method, and the zone diameters of each drug combination were never smaller than those produced by each drug alone.

Theoretically, the inhibition of fungal cell wall synthesis by echinocandins may enhance the cell membrane penetration of membrane active drugs [26]. Therefore, combination studies including echinocandins seem promising [27]. In studies evaluating the combination of CAS with VOR or AMB against various fungal isolates (*Candida, Aspergillus, Trichosporon*), promising results have been observed and antagonism has not been detected in general [28-32]. However, Elefanti et al. showed that serum had a major impact on synergistic interactions of echinocandin with AMB and VOR combinations against *Aspergillus* spp.; in the presence of serum, the synergistic interactions were reduced [33]. Thus these results should be confirmed with clinical studies.

In the present study, a noticeable alteration was not detected by Etest combination method in both ways according to MIC values of each drug alone. Although there are no criteria for

interpretation of combination tests by disk diffusion method, zone diameters of each drug combination were never smaller than those produced by each drug alone for the first two combination ways. In addition, we did not observe any result suggesting an antagonistic interaction, thus the alterations in the third disk diffusion combination way did not distinctive.

The common point of studies evaluating the CAS+VOR or CAS+AMB activities is that obvious antagonism has not been observed in none of them. We evaluated the CAS + VOR and CAS+AMB combinations against 30 clinical *C. krusei* by Etest and disk diffusion methods and we did not observe markedly antagonistic interaction.

4. CONCLUSION

Etest and disk diffusion can be easy, convenient, and nontime-consuming alternative methods to evaluate the antifungal combinations. Nevertheless, there are deficiencies with both application and interpretation periods of disk diffusion tests. Thus, further comparative studies are required for determining the reliability and the accuracy of these tests. The combinations of CAS with VOR and AMB have exhibited promising results because of the apparent antagonistic interaction was not detected. Combination of these antifungals may be considered for special clinical settings. However, these are in vitro results and should be confirmed by in vivo clinical studies.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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