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Efficacy of a fixed combination of three antibiotics against *Candida* species

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PURPOSE

Ophthalmic mycoses are being increasingly recognized as an important cause of morbidity and blindness. Keratitis due to yeasts is most frequently caused by *Candida albicans*. In this form of mycotic keratitis one or more ocular (e.g. insufficient tear secretion, defective eyelid closure, contaminated contact lenses) or systemic (e.g. diabetes mellitus, immunosuppression) conditions predispose to the infection. Treatment of fungal corneal ulcer mainly depends on readily available anti-fungal agents. Up to date, natamycin is the only one medicament commercially available for fungal keratitis (Thomas, 2003). Improvement of the antifungal arsenal is needed since existing antifungals can be associated with limited efficacy, toxicity and resistance. Recent scientific studies have reevaluated old antibiotics such as chloramphenicol, tetracyclines and polymyxins, traditionally used for bacterial infections, for their potential antifungal activity (Pankey et al., 2014; Joseph et al., 2015; Moullan et al., 2015).
Based on literature and clinical experience, we evaluated a widely used antibacterial ophthalmic combination (Colbiocin-AC) containing tetracycline, chloramphenicol and colistimethate sodium, in the fixed ratio 1:1:3, using *in vitro* tests and *ex vivo* keratitis models.

METHODS

In vitro

Candida albicans ATCC 2091 and 10231 and 14 clinical isolates of *C. albicans* (7), *C. glabrata* (5), *C. utilis* (1) and *C. tropicalis* (1) were used *in vitro* assays. The MICs of each antibiotic included in the AC (tetracycline, chloramphenicol and colistimethate sodium- idrosoluble salt of colistin) were determined either individually or in combination, in the fixed ratio 1:1:3, for all strains according to guidelines of NCCLS (Wayne, 2002). Fluconazole was tested for comparison. The Fractional Inhibitory Concentration (FIC) index was also calculated according to the following mathematical formula:

FIC index = (Ac/A)+(Bc/B)+(Cc/C)

A, B, C = are MICs of tetracycline, chloramphenicol and colistimethate sodium respectively.
Ac, Bc, Cc = Inhibent Concentrations of each compound in the fixed association.
A FIC index ≤ 0.5 indicates synergism, values between 0.5 and 2.0 indicate additivity/indifference, and values ≥ 2.0 indicate antagonism.

Time-killing curves for *C. albicans* ATCC 10231 and for C. albicans n. 4 (ocular isolate) were performed at 10 x MIC values of AC and fluconazole. The results are expressed as means ± standard deviation from three experiments and statistically analyzed by a one-way ANOVA, followed by *Tukey* post-test by GraphPad Prism Software. Differences in groups and treatments were considered significant for $p<0.05$.

Ex vivo

C. *albicans* ATCC 10231 and C. albicans n. 4 were used **in *ex vivo* rabbit keratitis model** (Marino et al., 2015). Twenty-four enucleated eyes, obtained from a local abattoir, were randomly divided into 2 groups (12 corneas/group) corresponding to *C. albicans* strains. Each group was **intrastromally injected with 50 µl** of yeast suspension containing 5 x 10^4 colony forming units (CFU)/ml. Two hours after the injection, corneas were divided into 3 groups of 4 corneas each. The groups were treated with **six instillations each of AC** (first-group), **fluconazole** (second-group) or **balanced salt solution** (third-control group) up to 24 h after injection. Then the tissues were homogenized and plated to determine the number of recovered CFU/g. The results are expressed as means ± standard deviation from three experiments and statistically analyzed by a one-way ANOVA, followed by Bonferoni post-test by GraphPad Prism Software. Differences in groups and treatments were considered significant for $p<0.05$.

***Ex vivo* keratitis model**

Fig. 1. Rabbit eye.

Fig. 2. Intrastromal injection of *C. albicans* suspension.

Fig. 3. Infected sclero-corneal buttons in culture.

RESULTS

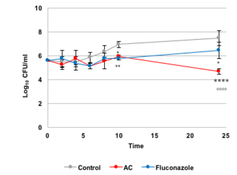
In vitro efficacy

MIC values for the individual antibiotics against all strains of *Candida sp.* used were higher than that observed for reference fluconazole. **Concurrently when the AC was used it was observed a reduction of the concentration of each antibiotic able to inhibit the yeast growth**. In 88% of strains tested the FIC index was between 0.5 and 2.0, indicative of an additive effect (Table 1). Only in the strains of *C. glabrata* and *C. tropicalis* the FIC index was < 0.5 or > 2.0 respectively, indicating a synergic or antagonist effect (Table 1). The Time-killing curves showed that the AC and fluconazole, at a concentration 10 x MIC were able to maintain under control the charge for *C. albicans* ATCC 10231 and *C. albicans* n° 4 up to 10 h. After 24 h, the AC reduced of 1 Log the charge compared to the inoculum at the 0 time point (Fig. 4 - Fig. 5). This effect was statistically superior to that of fluconazole.

Table 1. MIC and FIC index.

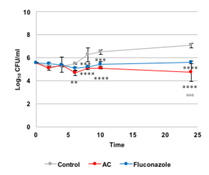
Strains	MIC µg/ml				FIC index
	Chloramphenicol	Tetracycline	Colistimethate sodium	Fluconazole	AC (Ratio 1:1:3)
C. albicans ATCC 10231	320	80	320	1.25	1.17
C. albicans ATCC 2091	160	160	160	1.25	1.39
C.albicans 4	320	160	320	0.625	0.85
C.albicans 15	160	40	80	0.31	1.58
C.albicans 12	160	80	160	1.25	1.705
C.albicans 13	320	160	320	0.625	1.706
C.albicans 16	320	320	320	2.5	1.39
C.albicans 355	320	80	320	2.5	1.17
C.albicans 18	320	80	320	2.5	1.17
C.glabrata 1	320	320	320	2.5	1.39
C.glabrata 3	320	160	320	1.25	0.43
C.glabrata 8	320	320	80	0.625	1.94
C.glabrata 9	320	40	320	1.25	1.785
C.glabrata 10	320	160	320	2.25	1.705
C. utilis 1	320	80	320	2.5	1.16
C. tropicalis 1	320	160	160	0.625	2.5

Fig. 4. Time-killing curve of C. *albicans* ATCC 10231.



**** : p < 0.0001 *vs* control
** : p < 0.01 *vs* control
* : p < 0.05 *vs* control
**** : p < 0.0001 *vs* fluconazole

Fig. 5. Time-killing curve of C. *albicans* 4.

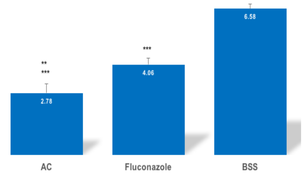


**** : p < 0.0001 *vs* control
*** : p < 0.001 *vs* control
* : p < 0.01 *vs* control
*** : p < 0.001 *vs* fluconazole

Ex vivo efficacy

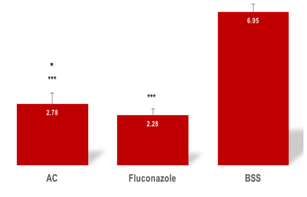
The AC was effective in the *ex vivo* rabbit keratitis experiments in decreasing the load of *C. albicans* ATCC 10231 and C. albicans n. 4. **AC reduced the both loads of *C. albicans* ATCC 10231 and *C. albicans* n. 4 by 4 $\log_{10}$ CFU/g respect to BSS (control) after six doses up to 24 h after infection**. Moreover, AC showed higher activity than fluconazole against *C. albicans* ATCC 10231 (ca 1 Log_{10} CFU/g) and a similar efficacy against *C. albicans* 4. The results are reported in Fig. 6 and Fig. 7. Moreover, AC treatment kept under control the corneal opacity better than fluconazole and BSS (Fig. 8).

Fig. 6. Efficacy of AC treatment against *C. albicans* ATCC 10231.



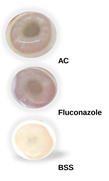
Mean Log_{10} CFU/g (± standard deviation) change in *C. albicans* ATCC 10231 loads of AC and fluconazole treated group *vs* BBS (control group) in corneal tissue. ****p* < 0.001 *vs* control; ***p < 0.01 vs* fluconazole.

Fig. 7. Efficacy of AC treatment against *C. albicans* 4.



Mean Log_{10} CFU/g (± standard deviation) change in *C. albicans* 4 loads of AC and fluconazole treated groups *vs* BBS (control group) in corneal tissue. ****p* < 0.001 *vs* control; **p >0.05 AC vs* fluconazole.

Fig. 8. Corneas after treatments.



Images showed a significant differences in the corneal opacity among groups.

CONCLUSIONS

Since fungi are eukaryotic cells, they share many pathways with human cells, thus increasing the probability of antifungal activity of "non fungal drugs". The AC (fixed antibiotic combination containing chloramphenicol, tetracycline and colistimethate sodium) showed good activity against *Candida* species *in vitro* and high efficacy against *C. albicans* in *ex vivo* keratitis. The underlying mechanism of this combined therapy may be explained by the following points: 1. tetracyclines and chloramphenicol promote mitonuclear protein imbalance and mitochondrial dysfunction (Moullan et al., 2015); 2. polymyxins bind lipopolysaccharide and anionic phospholipids in the bacterial cell membrane, disrupting membrane integrity (Pankey et al., 2014). It is possible that polymixins, such as colistin, act similarly against the fungal cell membrane. We showed that tetracycline, colistimethate sodium and chloramphenicol used alone inhibit the growth of yeasts at very high concentrations respect to fluconazole. **However, when the three antibiotics are used in combination (fixed ratio 1:1:3) an additive effect for the majority of *Candida* strains tested is noted. Probably, colistimethate sodium increases the permeability of the yeast membrane to tetracycline and chloramphenicol, which then inhibit mitochondrial protein synthesis. This hypothesis about their mechanism of action in combination has to be better investigated using specific assays. In conclusion, based on our *in vitro* and *ex vivo* results, we suppose that AC, already widely used in eye drop/ointment to treat bacterial eye infections, has the potential to be used clinically to treat fungal keratitis.**

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