Article

**Candida albicans ERG11 gene polymorphism: impact on its in vivo virulence and susceptibility to fluconazole according to the Galleria mellonella model**

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**Abstract:** (1) **Background:** The aim of this study is to have an idea on the molecular mechanisms of *C. albicans* resistance to fluconazole in Burkina Faso, by studying the polymorphism of the ERG11 gene, and its implication in the *C. albicans* virulence and resistance in vivo according to the *Galleria mellonella* model; (2) **Methods:** Ten (10) clinical strains including, 5 resistant and 5 susceptible and 1 virulent and susceptible reference strain SC5314 are used. For the estimation of virulence, the larvae were inoculated with 10 μL of *C. albicans* cell suspension at variable concentrations: 2, 5.10^5, 5.10^6, 1.10^7, and 5.10^6 CFU/larva of each strain. For the in vivo efficacy study, fluconazole was administered at 1, 4 and 16 mg/kg respectively to *G. mellonella* larvae, after infection by inoculum 5.10^6 CFU / larvae of each strain. (3) **Results:** Six (6) non-silent mutations in the ERG11 gene (K143R, F145L, G307S, S405F, G448E, V456I on ERG11p) were found in 4 resistant isolates. Larval mortality depended on fungal burden and strain. The inoculum 5.10^6 CFU caused 100% mortality in 2 days for the 2 CAAL-1 and CAAL-2 strains carrying the F145L mutation, in 3 days for the reference strain SC5314, in 4 days for the ensemble of resistant strains, and in 5 days for the ensemble of susceptible strains. The comparison of the mortality due to the reference strain SC5314 CFU / larva and the average mortality due to the two mutant F145L strains, shows a significant difference (P <0.05). Fluconazole significantly protected (P> 0.05) the larvae from infection by susceptible strains and the reference strain. However, 100% mortality in 6 days after injection of the resistant strains, was observed (4) **Conclusions:** Certain mutations in the ERG11 gene such as the F145L mutation are thought to be a source of increased virulence in Candida albicans. Fluconazole effectively protected larvae from infection by susceptible strains in vivo, unlike resistant strain.

**Keywords:** Burkina Faso; Candida albicans; ERG11; virulence; susceptibility; Fluconazole; Galleria mellonella
1. Introduction

*Candida albicans* is the most common opportunistic fungal pathogen in humans. It is a yeast responsible for diseases called candidiasis, ranging from infections of the superficial mucous membranes to fatal systemic disorders [1, 2, 3]. The treatment of candidiasis requires antifungal drugs. Among the available antifungals, azoles, in particular fluconazole (FCL), are commonly used, in the treatment of systemic candidiasis, because, their safety of use and the availability in oral and intravenous formulations [4, 5]. However, the implementation of long-termazole treatment such as for example in the HIV-infected patient with oropharyngeal candidiasis is a favorable situation for the development of resistance with various mechanisms [6]. Among the many mechanisms involved in *C. albicans* resistance to azoles, ERG11 gene (coding for sterol 14α-demethylase, azole target enzyme) non-silent mutations, is one of the most important mechanisms, both in terms of prevalence and in terms of impact on *C. albicans* susceptibility to these antifungals [6]. Currently, more than 140 mutations in the ERG11 gene have been reported in the literature and new mutations are regularly described illustrating the highly polymorphic nature of this gene [7].

In this study, our objective was to explore the ERG11 gene polymorphism in a *C. albicans* clinical isolates collection presenting different profiles of susceptibility to fluconazole (susceptible and resistant strains) in a first part. In a second part it was to know if the resistance found *in vitro* was also found *in vivo* on the one hand, and on the other hand, if the ERG11 gene mutations would increase or would decrease the virulence of the strains, in comparison with a reference strain. For the implementation of this second part, we used the insect *Galleria mellonella* model, the use of which has many advantages compared to mammalian models. Indeed, *G. mellonella* or wax moth is a species of lepidoptera (butterflies) found in most parts of the world [8]. The larva (caterpillar) of *G. mellonella* is of practical size (2 to 3 cm long), easy to use for experiments [9]. Its immune system is comparable to that of vertebrates because it has both a cellular and humoral immune response to infection. In addition [10] the larvae can be kept at temperatures between 15 °C and 37 °C [11], which makes the larvae well suited for studying pathogens at human body temperature [8]. These advantages make *G. mellonella* an attractive host for studying pathogens and antimicrobial agents [9, 12, 13].

2. Materials and Methods

*C albicans* strains used and culture media

A total of 10 clinical strains including 5 FCL susceptible strains and 5 FCL resistant strains were used. A reference strain SC5314 ((Institut für Molekulare Infektionsbiologie, Universität Würzburg, Würzburg, Germany), virulent and susceptible to FCL, was used as a control strain. The different strains were seeded in YPD medium (1% of extract of yeast, 2% peptone and 2% dextrose) + Ampicillin, Kanamycin and Chloramphenicol and incubated at 30 °C in a shaking incubator followed by subculturing in Sabouraud + chloramphenicol medium.

Antifungal susceptibility test

Susceptibility to FCL was confirmed for each isolate using the standard broth microdilution method recommended by the CLSI (Clinical and Laboratory Standards Institute), document M27-A3 (National Committee for Clinical Laboratory Standards). *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as controls. The FCL was purchased from Sigma (Sigma Chemical Corporation). The MIC (Minimum Inhibitory Concentration) which is the lowest drug concentration resulted in 50% growth inhibition compared to growth in the control well was determined visually after 48 hours incubation at 35 °C. MIC values for FCL were compared to the CLSI interpretative guidelines on antifungal susceptibility testing. Thus, MICs ≤8 μg / mL were considered as susceptible (S), 16 to 32 μg / mL as susceptible dose-dependent (SDD) and ≥64 μg / mL as resistant (R) to FCL.

Molecular analysis

The total genomic DNA was obtained from colonies of the *C. albicans* different strains aged 48 hours, grown on Sabouraud + Chloramphenicol. The DNA was extracted and purified using the NucleoSpin® Tissue kit (Macherey-Nagel) according to the manufacturer's instructions. The open
reading frame of the complete ERG11 gene (1451 bp) was amplified with a PTC-100 thermocycler (MJ Research, Waltham, MA) using the primers previously described: ERG11ORF-F — GAAAGGGAATTCAATCG and ERG11ORF-R— TGTTAATCCAACTAAGTAAC [13 ]. The reaction mixtures contained 1 μmol / L of each primer, 10 μL of 5 × buffer, 2 mmol / L of MgCl2, 0.2 mmol / L of each deoxyribonucleoside triphosphate, 0.1 U of GoTaq® DNA polymerase (Invitrogen, USA) and sterile water to a final volume of 50 μL. The amplification parameters were as follows: initial denaturation at 96 ° C for 3 min followed by 35 cycles of annealing at 56 ° C for 1 min, elongation at 72 ° C for 2 min, and denaturation at 95 ° C for 1 min. The PCR products were purified using 7.5 M ammonium acetate (15 μL) and absolute ethanol (74 μL). The sequencing was carried out using a Big Dye sequencing kit (Applied Biosystems, United States), followed by purification using a resin of fine DNA quality Sephadex G-50 (GE Healthcare, United Kingdom ) in a MultiScreen column loader (Merck Millipore, United States), and analyzed by electrophoresis in an ABI3500 automatic sequencer (Applied Biosystems, USA) using additional primers [14]. The nucleotide sequences were assembled using BioEdit software version 7.2.524, using ClustalW25 and adjusted manually using MEGA software, version 626. For each strain, the entire sequence of the open reading frame ERG11 was compared to an ERG11 sequence previously described (accession number X13296) and originating from a strain sensitive to fluconazole [15]. Silent mutations have not been taken into account.

**In vivo strains virulence estimation**

The larvae of *G. mellonella* were kindly provided to us by the IRS2 of the University of Nantes (France). We then set up and maintained a perennial breeding of *G. mellonella* within the Parasitology-Mycology laboratory of the Training and Research Unit in Health Sciences (UFR SDS) of the Joseph Ki-Zerbo University of Ouagadougou (Burkina Faso). This breeding allowed us to acquire larvae having ideal constant characteristics for a good *in vivo* infection model. Once grown from the egg, the larva is in the growth phase and goes through several stages for two to three weeks before reaching the desired weight for the study model. Nutrition is important during this phase. The larvae are sorted regularly so that they do not form cocoons too quickly and therefore do not turn into pupae too soon.

Once the desired weight has been reached, the larvae have already partially slowed down their nutrition and are sorted in batches of 10 individuals of equal weight and distributed in petri dishes. The larvae are then inoculated with 10 μL of *C. albicans* cell suspension at variable concentrations of 2,5.10^5, 5.10^5, 1.10^6, and 5.10^6 colony forming unit (CFU) of each strain (clinical and reference strains). The inoculum is prepared with PBS (phosphate-buffered-saline). The required larva weight for the experiment is 280 to 350 mg / larva [16]. The injections are made at the last right or left proleg with a fine needle insulin syringe. To verify that the volume injected has no negative effect on the larvae, we previously studied the survival of the larvae after five repetitive injections of 10 μl phosphate buffered saline (PBS) for 2 days, compared to the non-injected larvae.

The injected larvae are kept in the dark at 37 ° C (temperature close to that of the human body) and without food. Mortality as well as melanization (generally occurring as early as 30 minutes after inoculation) were observed every 24 hours for 12 days [16]. The larva was considered dead when there was no movement following gentle pressure from the cuticle. Dead larvae are removed from the Petri dish housing the remaining viable larvae.

The average of mortality was calculated per day for each type of strain (susceptible strains and resistant strains) and for each inoculum. The data obtained were used for the determination of the survival curves. A strain is considered to be virulent if it causes 100% mortality of the larvae in 120 hours maximum and at a given value of CFU inoculated [16].

**In vivo Study of strains susceptibility to FCL**

Inoculum 5.10^6 CFU / larva was used for the FCL efficacy tests according to the protocol defined by De-Dong et al. in 2013 [16]. This inoculum having caused a mortality of 100% of the larvae in 4 days concerning our reference strain. The FCL powder, was dissolved in physiological water. It was then administered at 1, 4 and 16 mg / kg respectively to the last left proleg, 30 minutes after infection in the right proleg, by the inoculum 5.10^6 CFU / larva [16] of each strain. Physiological water is injected into a control lot to ensure that mortality is not due to physical injury, or infection with
pathogenic contaminants. A second control group was infected with inoculum 5.10\(^6\) CFU / larva and without receiving subsequent treatment with FCL. As in virulence tests, mortality is monitored every 24 hours for 12 days [9]. Average of mortality was calculated per day for each type of strain (susceptible and resistant strains) for each dose of FCL. The data obtained were used for the determination of the survival curves.

For the study of virulence as for the study of the FLC efficacy, the mortality curves were plotted and examined using the Kaplan-Meier method on XLSTAT 2019.1.2 (Addinsoft) for EXCEL. The differences were determined using the log-rank test. The difference was considered significant for the P value <0.05.

Ethical considerations

Patients included for the clinical strains collection, received a clear explanation of the study objectives and gave their informed consent before participating in the study. The protocol for this thesis work has received the approval of the national ethics committee for health research in Burkina Faso (Deliberation No 2015-4-041).

3. Results

A total of 10 clinical strains including 5 FC susceptible strains and 5 FCL resistant strains were used. A reference strain SC5314, virulent and FLC susceptible was used as a control strain.

3.1. Analysis of amino acid substitutions on Erg11p

The amplification of the complete \(\text{ERG11}\) gene and the search for amino acid substitutions were carried out for each of the 10 clinical isolates and for the reference strain. As reported in previous studies, we encountered silent mutations in 3 of 10 clinical strains. No mutation was observed with 4 strains including the reference strain SC5314. The 4 remaining strains carried mutations in the \(\text{ERG11}\) gene and were all resistant to FCL. The number (n) of amino acid substitutions on \(\text{Erg11p}\) varied between the strains, CAAL-1 (n = 2), CAAL-3 (n = 1) and CAAL-6 (n = 3) and CAAL-7 (n = 1). A total of 6 distinct types amino acid substitutions have been identified, all of which have been previously described as associated with resistance to FCL (MIC > 64 µg / mL). These are: K143R, F145L, G307S, S405F, G448E, V456I (Table 1).

Table 1: Distribution of the different amino acid substitutions at Erg11p in the different strains

| Strains | Isolation site | FCL MIC(µg/mL) | Amino acid substitution on Erg11P |
|---------|----------------|----------------|---------------------------------|
| CAAL-1  | oral           | > 64           | K143R, F145L                   |
| CAAL-2  | oral           | < 0,125        | None                           |
| CAAL-3  | oral           | > 64           | G307S                          |
| CAAL-4  | oral           | < 0,125        | None                           |
| CAAL-5  | Fecal          | > 64           | None                           |
| CAAL-6  | Fecal          | > 64           | S405F, G448E, V456I            |
| CAAL-7  | Fecale         | > 64           | F145L                          |
### 3.2. In vivo virulence estimation

All *G. mellonella* larvae melanized within 30 minutes after strain inoculation regardless of strain and inoculated fungal burden (Figure 1).

![Figure 1: Evolution of G. mellonella larvae 30 minutes after infection](https://example.com/image)

*Source: Laboratory of Parasitology-Mycology UJKZ*

The larvae melanization, evidence of the activation of the immune system in response to the infection, occurred independently of the fungal strain injected and the concentration of the inoculum.

With the PBS injection, we did not observe any melanization or mortality. All larvae receiving the PBS injection survived after 12 days of observation (Figure 2).
The observed mortality depended on the fungal burden and the strain. Regarding the inoculum 1, 25.10^5 UFC/larva, 12 days after infection, no mortality was observed. While with the mutant CAAL-7 strain F145L we observed 10% mortality on the 11th day with the inoculum 1, 25. 10^5. 100% mortality of the larvae was observed with inoculum 5.10^6 of the reference strain SC5314 in 72 hours (Figure 3 A). For the same inoculum, and concerning the ensemble of the susceptible strains, 100% mortality was noted in 120 hours (Figure 3B). As for the ensemble of the resistant strains and always for the same inoculum, 100% mortality was observed earlier, in 96 hours (Figure 3C).
Figure 3: Evolution of larval survival after inoculation of: (A) reference strain SC1453; (B) ensemble of susceptible strains (SS); (C) ensemble of resistant strains (RS)

For all types of strains (SC5314, SS and RS), the comparison of survival was made with the batch of larvae which received the injection with PBS (Phosphate - Buffered-Saline). Survival in the batch which received the $2.5 \times 10^5$ CFU / larva inoculum is similar to that in the PBS batch ($P = 1.000$) regardless of the type of strain. Almost identical finding with inoculum $5.10^5$ CFU / larva. Concerning inoculum $5.10^6$ CFU / larva, the difference compared to the PBS batch was significant for all type of strains ($P < 0.0001$).

The two strains CAAL-1 and CAAL-7 carrying the F145L mutation and resistant to FCL appeared to be more virulent than the rest of the strains. The evidence of the increased virulence of these two mutant F145L strains was especially remarkable with the inoculum $5.10^6$ CFU/larva. With this inoculum, each of these two strains resulted in 100% mortality of the larvae in 48 hours.

The comparison of the virulence of the reference strain SC5314 with the other types of strains for the inoculum $5.10^6$ CFU, showed a non-significant difference in mortality between the reference strain SC5314 and the ensemble of susceptible strains ($P = 0.139$) (Figure 4). The observation is the same between the reference strain SC5314 and the ensemble of resistant strains ($P = 0.869$) (Figure 4). However, still with inoculum $5.10^6$, the comparison of the mortality due to the reference strain SC5314 CFU/larva and the average mortality due to the two mutant F145L, shows a significant difference ($P = 0.009$) (Figure 4).
Figure 4: Evolution of the survival of the larvae after injection of the reference strain SC5314, of ensemble of the susceptible strains (SS), of ensemble of the resistant strains (RS), of ensemble of the two resistant mutant F145L strains (SRm) with the inoculum $5 \times 10^6$ CFU/larva.

3.1. In vivo different strains susceptibility to fluconazole study

Inoculum $5 \times 10^6$ CFU/larva having led to the death of 100% of the larvae in 3, 4 and 5 days with respectively, the reference strain SC5314, the ensemble of the resistant strains and the ensemble of the susceptible strains, therefore constituted the ideal inoculum for studying the in vivo strains susceptibility to FCL. Our results indicate that FCL significantly protected ($P > 0.05$) the larvae from infection by susceptible strains and the reference strain which is also FCL susceptible. More precisely, with the 16 mg/kg dose injected, 30 minutes after infection of the strain SC5314, 80% of the larvae survived up to 12 days, our observation period (Figure 5A). With susceptible strains, 90% of the larvae survived after 12 days (Figure 5B). Antifungal protection of the larvae against infection was dose-dependent. However, the antifungal protection of the larvae after injection of the resistant strains proved to be poor. The difference between the number of larvae which survived after FCL administration following the inoculation of resistant strains, (whatever the dose) and the number of larvae which received the injection of resistant strains without FCL, was not significant ($P < 0.05$). No larvae survived after 12 days, 100% mortality in 6 days (Figure 5C).
Figure 5: Estimation of the \textit{in vivo} protective role of FCL after inoculation of the different strains with the inoculum $5 \times 10^6$ CFU: (A) \textit{in vivo} protective role of FCL on the reference strain SC 5314; (B) \textit{in vivo} protective role of fluconazole on the ensemble of susceptible strains; (C) \textit{in vivo} protective role of fluconazole on the ensemble of resistant strains.

The efficacy of FCL at high doses, 16 mg/Kg on the reference strain SC5314 was compared with its efficacy on the clinical strains. The efficacy of FCL on strain SC5314 and on the ensemble of susceptible strains (SS) were almost similar ($P = 0.591$) (Figure 6). However, the FCL was significantly less effective on the ensemble of resistant strains compared to the reference strain SC5314 ($P < 0.0001$) (Figure 6).
Figure 6: Estimation of the *in vivo* protective role of FCL at a dose of 16 mg/Kg after inoculation of the different strains with inoculum 5.106 CFU

4. Discussion

In this study, the mechanisms of 10 *C. albicans* clinical FCL resistant strains obtained from various isolation sites in patients of the CHU Yalagado Ouédraogo in Ouagadougou (Burkina Faso) were evaluated. This is the first preliminary molecular analysis of the *C. albicans* resistance to FCL in Burkina Faso. Our results indicate for the first time in Burkina Faso punctual mutations in the ERG11 gene, one of the molecular mechanisms involved in the *C. albicans* resistance to FCL in our clinical strains.

Although most *C. albicans* clinical isolates are susceptible to triazoles, including FCL, some authors have reported an increase in the incidence of invasive infections due to strains resistant to FCL[10,11,17]. The emergence of *C. albicans* resistance to FCL is a source of preoccupation due, on the one hand, to the frequency of often serious infections due to this fungal species, and, on the other hand, to the use of FCL as a medicine of first choice in care [18]. Although mutations in the *ERG11* gene are consistently reported as one of the main mechanisms of resistance in *C. albicans*, resistance to FLC has been shown to be due to a combination of different molecular mechanisms [19, 20, 21]. The literature generally describes four mechanisms of *C. albicans* resistance to FCL and in general, to azoles what are:

The modification of the FCL action target, 14-alpha-demethylase that corresponds to amino acid substitutions in the protein sequence [18, 22]. These modifications, which are the consequence of punctual mutations in the *ERG11* gene coding for 14-alphademethylase [23, 24] lead to a decrease of FCL affinity for 14-alpha-demethylase, or to changes in conformation, prevent the access of the antifungal agent active site [22]. According to Carvalho et al. in 2014, more than 140 resistant mutations were described on the *C. albicans* *ERG11* gene [25]. This is why, according to the literature, several authors have been particularly interested to this gene in the various studies on the *C. albicans* resistance to azoles;

Overproduction of the action target: By increasing the production of the target enzyme, 14-alphademethylase, *C. albicans* may decrease its susceptibility to theazole antifungals activity o [17,19, 24].
Efflux phenomena: To exercise their antifungal activity, azoles must enter the fungal cell and be at a sufficient intracellular concentration to inhibit 14-alpha-demethylase [19]. *C. albicans* has, naturally, at its plasma membrane, transporters called efflux pumps allowing the efflux of different molecules [18]. The CDR1, CDR2 and MDR1 genes code for these efflux pumps which are membrane transporters which excrete toxic molecules out of the fungal cell. CDR1 and CDR2 code for the ABC transporters, and the MDR1 gene codes for the MFS transporters [18]. Increased expression of these transporters (efflux pumps) is an important *C. albicans* clinical strains mechanism of resistance to azoles [19];

Alterations in the ergosterol biosynthesis pathway: in the presence of azoles, the inhibition of 14-alpha-demethylase leads to the accumulation of methylated sterols which are transformed into toxic products by delta-5, 6-desaturase, an enzyme encoded by the ERG3 gene [26, 27]. If the ERG3 gene is mutated, these toxic products are no longer synthesized and the fungal cell can survive and thus become resistant to azoles [26]. This resistance mechanism, although uncommon, has been demonstrated in certain *C. albicans* clinical strains [26].

In this study, four of the five *C. albicans* isolates with high (resistant) FCL MICs had one or more punctual mutations in the ERG11 sequence which led to six amino acid substitutions: K143R, G307S, S405F, G448E, V456 and, F145L, compared to the reference strain SC5314. New point mutations in the ERG11 gene responsible for resistance have been reported by various authors [6,7]. However, in the present case, all the six mutations identified have already been described in the literature [28, 29, 30].

In order to verify a possible impact of non-silent mutations, on the virulence of the strains concerned, we found that the mutant CAAL-1 and CAAL-7 F145L strains seem more virulent compared to the reference strain and other clinical strains (susceptible and resistant) according to the *C. albicans-G. mellonella* infection model. This suggests that the F145L mutation is associated with an increase of the virulence of the strain which carries it. Although the F145L mutation has already been demonstrated in the literature, its involvement in increasing virulence has never been studied. In addition, this finding of increased virulence due to the F145L mutation suggests that certain genetic mutations in the ERG11 gene and probably in other genes in *C. albicans* could not only generate resistance, but could also be a source of increased virulence. However, considering the mortalities due to the different types of strains as a whole, the average larval mortality was 100% in 3 days with the reference strain SC5314, in 4 days with the ensemble of resistant strains, and in 5 days for the ensemble of susceptible strains with inoculum $5 \times 10^6$ CFU/larva(Figure 3). No significant difference in mortality was observed between the different types of clinical strains as a whole compared to the reference strain SC5314 (Figure 4).

To study the FCL in vivo efficacy, larvae of *G. mellonella* were infected to assess the response to FLC therapy. In vitro Resistance to FCL has been well confirmed in vivo. Resistant strains in vitro behaved significantly the same way in vivo, causing the death of all larvae in 4 days despite the FCL administration, regardless of the dose administered. As for susceptible strains in vitro, these also significantly ($P>0.05$) showed their susceptibility in vivo following the administration of fluconazole. Several authors having carried out work on the same subject had arrived at the same observation [31, 32, 33].

In perspective, this study should be continued in order to detect other molecular mechanisms involved in the resistance of *C. albicans* to azoles in general and in particular to FCL. These include the overexpression of the ERG11 gene, MDR1 and CDR1. In addition, the implication of certain resistant mutations, as one of the sources of increased virulence, must be further elucidated through the study of the various determinants of virulence in the strains of *C. albicans* concerned.

5. Conclusions
ERG11 gene mutations are involved in the resistance of C. albicans to FLC. In addition, some of these point mutations in the ERG11 gene would increase the virulence of the mutant strains compared to the reference and wild-type strains according to the C.albicans-G. mellonella infection model. In our study, the observation in this sense was made with the F145L mutation.

Author Contributions:
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Zida Adama: Conceptualization, XX and YY; Validation, XX; Resources, XX
Soulama Issiaka: Validation, XX; Resources, XX,
Samuel S Sermé: Investigation, XX; Resources, XX,
Thierry K Guiguemdé: Writing - Review & Editing, XX
Ibrahim Sangaré: Writing - Review & Editing, XX
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