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Comparison of molecular techniques for identification of *Fusarium* species from clinical samples.

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REVISED ABSTRACT

Background: The genus *Fusarium* emerged over the past three decades as an opportunistic pathogen of immunocompromised hosts, mainly those with persistent and severe neutropenia. The early diagnosis of infection may optimize the antifungal therapy and be crucial for patient recovery. Nucleic acid based methods are often used to identify *Fusarium* spp. This study aimed to compare the techniques of loop-mediated isothermal amplification (LAMP), Real Time PCR and DNA sequencing for the identification of *Fusarium* spp. isolated from blood culture of patients with onco-hematologic disease from University Hospital at Unicamp.

Methods: Sixteen *Fusarium* isolates recovered from patients between 2006 and 2012 were selected. All isolates have been previously identified through classic methods (macroscopic and micromorphological characteristics) by the Clinical Mycology Laboratory. The LAMP technique consisted of three pairs of primers that identified six distinct regions of the gene *RBP2*. The Real Time PCR was performed in order to identify *Fusarium solani* species and *Fusarium* non-*solani species* complex using 28s ribosomal RNA gene. The sequencing was performed in an ABI Prism Genetic Analyzer 3100 (Applied Biosystems) using *EF1α* gene amplification and BigDye® (Applied Biosystems) reagent, according to manufacturer instructions. The sequences were assembled using ATSQ version 6.0.1 (Genetix) and compared with database information available at National Center for Biotechnology Information (NCBI).

Results: Fourteen of the isolates were identified as belonging to *Fusarium solani* species complex and two as *Fusarium* non-*solani* species complex using LAMP and Real Time PCR methodologies. The sequencing analysis identified fourteen isolates as *Fusarium solani* species complex and two as *Fusarium napiforme* (non-*solani*). The three methodologies showed 100% of specificity and sensitivity and 100% of concordance among them.

Conclusion: Considering the high performance of the three methodologies we conclude that LAMP is more accessible when compared with sequencing and real time PCR techniques, since it shows a lower cost and faster runtime. These results indicate LAMP as a promising methodology that can be used for filamentous fungi routine diagnosis, assisting in make decision for appropriate therapy of patients.

INTRODUCTION

•The genus *Fusarium* emerged over the past three decades as an opportunistic pathogen of immunocompromised hosts, mainly those with persistent and severe neutropenia.

•The early diagnosis of infection may optimize the antifungal therapy and be crucial for patient recovery.

•Nucleic acid based methods are often used to identify *Fusarium* sp.

OBJECTIVES

•Compare the techniques of loop-mediated isothermal amplification (LAMP), Real Time PCR and DNA sequencing for the identification of *Fusarium* spp. isolated from bloodculture of patients with onco-hematologic disease from University Hospital at Unicamp.

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METHODS AND ISOLATES

•**Setting**: Section of Mycology, Clinical Laboratory, University Hospital and Laboratory of Molecular Epidemiology and Mycosis, Faculty of Medical Sciences, Campinas State University; Sao Paulo, Brazil.

•**Design:** Retrospective cohort.

•**Selection of isolates:** Sixteen *Fusarium* spp. isolates were selected from 2006 to 2012. All isolates have been previously identified by classical methods (macroscopic and micro morphological characteristics) by the Clinical Mycology Laboratory

•**Methods:** The DNA extraction was performed by the alkali lysis method, according to Genes Verocytotoxin typing PCR (Takara) manual kit. Later, DNA samples were submitted to the techniques described below:

• **Loop-mediated isothermal amplification (LAMP):** in brief, the reaction consists of three pairs of primers ,designed in the software PrimerExplorer (1), that identified six distinct regions of the gene *RPB2* in the regions ITS1 and ITS 2 at a constant temperature of 63°C. The products of LAMP reaction were observed in a real-time turbidity meter (Loopamp EXIA; Eiken Co., Japan) **(Figure 1).**

•**Real Time PCR** was performed in order to identify *Fusarium solani species* and *Fusarium* non-*solani* species complex using 28s ribosomal RNA gene, in the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) **.**

•**Sequencing** was performed in an ABI Prism Genetic Analyzer 3100 (Applied Biosystems) using *EF1α* gene amplification and BigDye® (Applied Biosystems) reagent, according to manufacturer instructions. The sequences were assembled using ATSQ version 6.0.1 (Genetix) and compared with database information available at NCBI bank (National center for biotechnology information) **(Figure 3).**

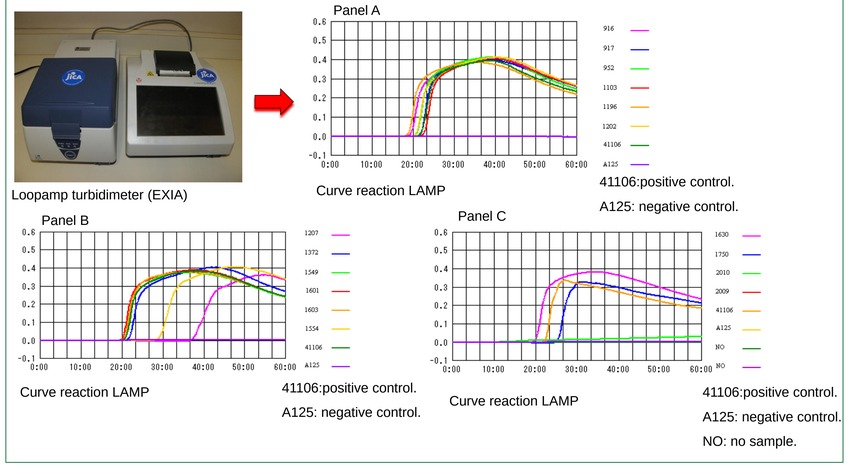
RESULTS:

Table 1. Comparison between the three methodologies

Nº LIF Isolates	Identification LAMP	Identification Real Time PRC	Identification DNA Sequencing	Agreement %
916	FSSC	FSSC	FSSC	100%
917	FSSC	FSSC	FSSC	100%
952	FSSC	FSSC	FSSC	100%
1103	FSSC	FSSC	FSSC	100%
1196	FSSC	FSSC	FSSC	100%
1202	FSSC	FSSC	FSSC	100%
1207	FSSC	FSSC	FSSC	100%
1372	FSSC	FSSC	FSSC	100%
1549	FSSC	FSSC	FSSC	100%
1554	FSSC	FSSC	FSSC	100%
1601	FSSC	FSSC	FSSC	100%
1603	FSSC	FSSC	FSSC	100%
1631	FSSC	FSSC	FSSC	100%
1750	FSSC	FSSC	FSSC	100%
2010	FNSSC	FNSSC	*F . napiforme* (FNSSC)	100%
2009	FNSSC	FNSSC	*F . napiforme* (FNSSC)	100%

Nº LIF : isolate number of Infection Fungal Laboratory; FSSC :(*Fusarium solani* species complex); FNSSC: (*Fusarium* non-*solani* species complex).

Figure 1. Equipment Loopamp turbidimeter (EXIA) and Curve reaction LAMP of equipment a real-time turbidity meter (Loopamp EXIA; Eiken)



DISCUSSION:

In our study the three methodologies identified the sixteen isolates of *Fusarium* spp. with 100 % of agreement , but the LAMP was faster and cheaper , showing more accessible than real time PCR and sequencing. Besides that, Lamp technique can amplify DNA with high specificity, efficiency and rapidity under isothermal conditions, resulting in an accumulation of 10^9 to 10^{10} target copies in less than an hour with four primers recognizing six distinct regions on the target. The amplification time can be further shortened by using an additional two primers - named the loop primers - and the products can be detected not only using specialized equipment but also by visual observation of turbidity or fluorescence. Furthermore, the sensitivity of LAMP appears not to be affected by the presence of non-target DNA in samples, neither by known PCR inhibitors such as blood, serum, plasma, or heparin(2,3). For this reason LAMP is a promising technique for the identification of *Fusarium*.

CONCLUSION

• The three methodologies showed high performance

•LAMP is more accessible when compared with sequencing and real time PCR techniques, since it shows a lower cost and quicker performance.

• These results indicate a promising methodology that can be used for filamentous fungi routine diagnosis, assisting in therapy and appropriate treatment of patients.

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