Quantitative mucormycosis PCR adapted to a fast platform for a same-day result

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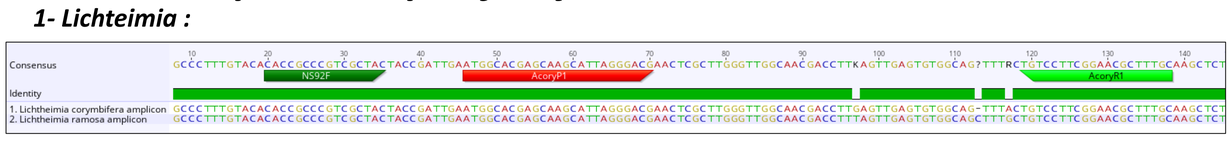
Abstract Fungi of the Mucorales order might be human opportunistic pathogen causing infection in immunocompromised patients with a high mortality rate. Conventional diagnostic consist in a macro- and micro-scopy observation when a positive culture is obtained. However, culture is often fastidious, and identification to the genus level might be somehow problematic and required trained people. Targeted PCRs for the four main Mucorales genera responsible for human infections so called, *Rhizopus, Mucor, Lichteimia*, and *Rhizomucor*, were shown to exhibit a higher sensitivity and allow accurate identification and quantification[1]. Until now, no such PCR were available at the CHUV and such a diagnostic was outsourced at the University Hospital of Besançon, France [1]. This outsourcing was time, and cost consuming, and not convenient for the clinicians in our hospital. We, therefore, decided to import the 3 PCRs (*Rhizopus-Mucor, Lichteimia*, and *Rhizomucor*) performed in University Hospital of Besançon, developed by Millon and collaborators [1]. However, our platform has a TAT of 4 hours using a Fast qPCR program completed in 45 min[2]. We thus had to adapt and validate the previously developed Mucorales PCR for a fast protocol.

I - Initial PCR carachteristics :

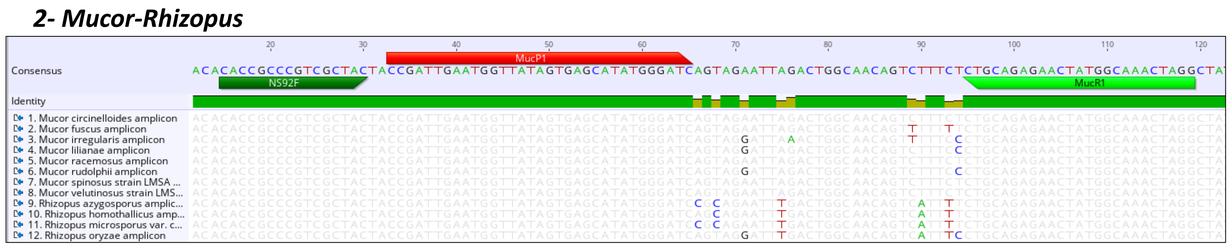
In this study, we used 3 triplets of oligonucleotides (2 primers and 1 probe) optimized by Million et al. [1] on a classical qPCR platform. One PCR detect *Lichtemia*, one *Mucor and Rhizopus* and the last one *Rhizomucor* specifically.

Probe and primers specificity:

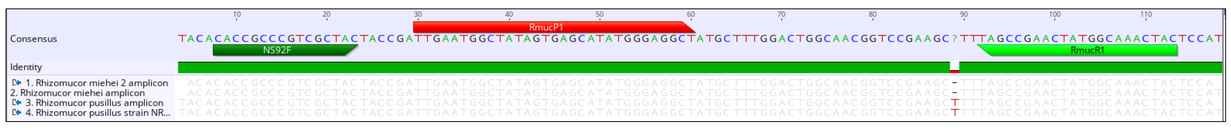
1- Lichteimia :



2- Mucor-Rhizopus

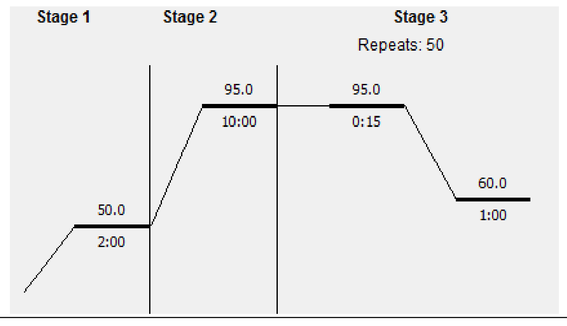


3- Rhizomucor



DNA was extracted from 1mL of sample, eluted in 50 µL, and 9 µL was used for qPCR

Initial PCR program : 2h15



Primers and probes final concentrations

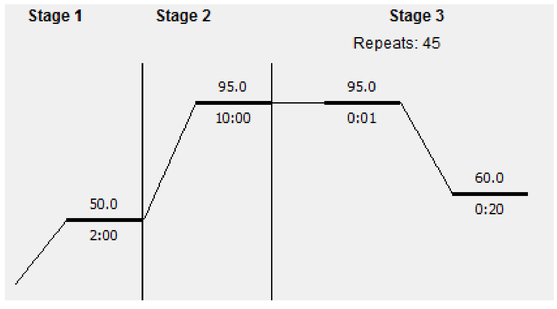
Probe : 0.08 µM
Primers : 1 µM

the PCR reactions are done with 9µl of DNA in a final volume of 20 µl

II - Characteristic of the Fast platform and problematic

DNA was extracted from 200 µL of sample, eluted in 100 µL, and 5 µL was used for each of the qPCR assays.

Fast-PCR program : 1h



➢ **The quantity of DNA used on the fast platform was much less than what was published by Millon et al.[1]**

➢ **Due to major PCR program modifications with the Fast program all PCR mix conditions have to be reset.**

Compatibility primers and probes on the Fast platform

Primer/probe	sequence	Tm °C (Santa-Lucia from Primer 3)	Ok on our «fast» platform
NS92-F	CACCGCCCGTCGCTAC	60.2	✓
Muc-R1	CCTAGTTTGCCATAGTTCTCTGCAG	61.4	✓
Acory-R1	GCAAAGCGTTCCGAAGGACA	61.5	✓
Rmuc-R1	GTAGTTTGCCATAGTTCGGCTA	58.2	✓
Muc-P1	CCGATTGAATGGTTATAGTGAGCATATGGGATC	67.5	☒
Acory-P1	ATGGCACGAGCAAGCATTAGGGACG	65.2	☒
RmucP1	TTGAATGGCTATAGTGAGCATATGGGAGGCT	67	☒

➢ **The 3 probes have a too low Tm to fit with the fast platform**

III - Optimisation of Fast-PCR conditions

Probe Tm optimisation :

Primer/probe	sequences	Tm °C (Santa-Lucia from Primer 3)	Ok on Fast platform
NS92-F	CACCGCCCGTCGCTAC	60.2	✓
Muc-R1	CCTAGTTTGCCATAGTTCTCTGCAG	61.4	✓
Acory-R1	GCAAAGCGTTCCGAAGGACA	61.5	✓
Rmuc-R1	GTAGTTTGCCATAGTTCGGCTA	58.2	✓
Muc-P1	CCGATTGAATGGTTATAGTGAGCATATGGGATC	67.5	☒
MUCO-LNA-P2-FAM	CCG+ATTG+AATGGTTATAGTGAGCATATGGGATC	69.5	✓
Acory-P1	ATGGCACGAGCAAGCATTAGGGACG	65.2	☒
LICHT-LNA-P1-FAM	AT+GGC+ACGAGCAAGCATTAGGGACG	69.2	✓
RmucP1	TTGAATGGCTATAGTGAGCATATGGGAGGCT	67	☒
RIMUC-LNA-P1-VIC	TT+GAATGGCTATAGTGAGCATATGGGAGGCT	69	✓

Nucleic acid with a + are Locked Nucleic Acids (LNA), which confer a higher Tm of the oligonucleotide.

Probes and primers PCR conditions:

- Quality criteria : -> 3 10-fold dilutions of a plasmid carrying the target sequence slopes between -3 and -4.
  -> ΔRn ideally around 1.
- Selected conditions :

organisms detected	PCR name	Final concentration (µM)		PCR metadata						
		Primers F and R	Probe	Ct T1000	Ct T100	Ct T10	slope	ΔRN T1000	ΔRN T100	ΔRN T10
Lichteimia	LI05	0.2	lna 0.1	28.00	31.08	35.27	-3.64	1.03	0.99	0.81
Rhizomucor	RMU08	0.7	n 0.3	29.02	32.07	35.51	-3.24	1.34	1.19	0.91
Mucor/Rhizopus	MU12	0.3	lna 0.2	29.18	32.20	36.21	-3.59	1.20	1.13	0.81

Lna : version of the probe with Locked nucleic acids ; n : probe version with classical nucleic acids

All the PCR reactions are done with 5µl of DNA in a final volume of 20 µl

IV - Analyses of the 3 Fast-PCRs performance

Contamination test:

As Mucorales could be present in the environment we checked that all steps of the workflow do not contaminate our analysis. For this purpose, PCR were done 20 times on negative samples (H2O) and 3 times on 5 differents extraction controls.

➡ **ALL NEGATIVE**

Specificity test :

The 3 PCRs were performed in duplicates on 20 other fungi or parasites DNA :

	Species	PCR LICHTEMIA	PCR MUCOR/RHIZOPUS	PCR RHIZOMUCOR
Aspergillus sp.	*Aspergillus fumigatus*	NEGATIVE	NEGATIVE	NEGATIVE
	Aspergillus niger	NEGATIVE	NEGATIVE	NEGATIVE
	Pneumocystis	NEGATIVE	NEGATIVE	NEGATIVE
Candida sp.	*Candida albicans*	NEGATIVE	NEGATIVE	NEGATIVE
	Candida glabrata	NEGATIVE	NEGATIVE	NEGATIVE
	Candida auris	NEGATIVE	NEGATIVE	NEGATIVE
	Candida krusei	NEGATIVE	NEGATIVE	NEGATIVE
	Candida parapsilosis	NEGATIVE	NEGATIVE	NEGATIVE
Misceallenous fungi	*Saccharomyces cerevisiae*	NEGATIVE	NEGATIVE	NEGATIVE
	Fusarium sp.	NEGATIVE	NEGATIVE	NEGATIVE
	Cladosporium sp.	NEGATIVE	NEGATIVE	NEGATIVE
	Histoplasma capsulatum	NEGATIVE	NEGATIVE	NEGATIVE
	Penicillium sp.	NEGATIVE	NEGATIVE	NEGATIVE
	Trichophyton rubrum	NEGATIVE	NEGATIVE	NEGATIVE
Toxoplasma	*Toxoplasmose*	NEGATIVE	NEGATIVE	NEGATIVE
Plasmodium sp.	*Plasmodium ovale*	NEGATIVE	NEGATIVE	NEGATIVE
	Plasmodium falciparum	NEGATIVE	NEGATIVE	NEGATIVE
	Plasmodium knowlesi	NEGATIVE	NEGATIVE	NEGATIVE
	Plasmodium vivax	NEGATIVE	NEGATIVE	NEGATIVE
Leishmania	*Leishmania infantum*	NEGATIVE	NEGATIVE	NEGATIVE

➡ **The 3 *Mucorales* PCR do not amplify any other species tested**

Sensibility test using plasmid positive control :

The PCRs were performed 5 times on 5 replicates of 5 10-fold dilutions plasmids : 10^3, 10^2, 10, 1 and 0.1 cp/ml

	PCR LICHTEIMIA			PCR MUCOR/RHIZOPUS			PCR RHIZOMUCOR		
plasmid (cp/ml)	Ct moy.	SD	% pos.	Ct moy.	SD	% pos.	Ct moy.	SD	% pos.
1.00E+03	27.92	0.15	100.00	28.83	0.35	100.00	29.58	0.49	100.00
1.00E+02	31.18	0.23	100.00	32.26	0.32	100.00	32.98	0.52	100.00
1.00E+01	34.69	0.52	100.00	35.94	0.73	100.00	37.39	3.79	96.00
1.00E+00	45.97	8.88	52.00	46.36	8.49	52.00	49.17	7.94	36.00
1.00E-01	55.00	0.00	0.00	53.67	4.61	8.00	55.00	0.00	0.00
Slope	-3.32	0.17		-3.17	0.16		-3.30	0.11	

➡ **The 3 *Mucorales* PCR have a 100 % sensibility at 100 copies /ml**

Test on known samples/strains:

Finally, the PCRs were tested on 20 known positive samples for Mucorales and on 20 samples negative for our Panfungal PCRs (18S or 26S rRNA):

POSITIVE SAMPLES :

ORIGIN	MATERIEL PCR	RESULTAT	IDENTIFICATION BY	PCR LICHTHEIMIA	Ct PCR LICHTHEMIA	PCR MUCOR/RHIZOPUS	Ct PCR MUCOR/RHIZOPUS	PCR RHIZOMUCOR	Ct PCR RHIZOMUCOR
EXPECTORATION	Strain 1000x diluted	*Rhizopus spp.*	microscopy	NEGATIVE	-	POSITIVE	26.6	NEGATIVE	-
CUTANEOUS BIOPSIE	Strain 1000x diluted	*Lichtheimia corymbifera*	26S sequencing	POSITIVE	30.4	NEGATIVE	-	NEGATIVE	-
NASAL SWAB	Strain 1000x diluted	*Mucor spp*	microscopy	NEGATIVE	-	POSITIVE	29.3	NEGATIVE	-
BRONCHOSCOPY	Strain 1000x diluted	*Rhizopus spp.*	microscopy	NEGATIVE	-	POSITIVE	24.4	NEGATIVE	-
EPIPLON FRAGMENT	sample	*Lichtheimia corymbifera*	26S sequencing	POSITIVE	20.95	NEGATIVE	-	NEGATIVE	-
SMALL INTESTINE FRAGMENT	sample	*Lichtheimia corymbifera*	26S sequencing	POSITIVE	22.9	NEGATIVE	-	NEGATIVE	-
BURN BACK SWAB	Strain 1000x diluted	*Lichtheimia corymbifera*	26S sequencing	POSITIVE	27	NEGATIVE	-	NEGATIVE	-
BURN HAND SWAB	Strain 1000x diluted	*Rhizopus oryzae*	26S sequencing	NEGATIVE	-	POSITIVE	23.4	NEGATIVE	-
BURN LEGS SWAB	Strain 1000x diluted	*Rhizopus oryzae*	26S sequencing	NEGATIVE	-	POSITIVE	26.5	NEGATIVE	-
NASAL BIOPSY	Strain 1000x diluted	*Rhizopus oryzae*	26S sequencing	NEGATIVE	-	POSITIVE	21.3	NEGATIVE	-
TISSUS FRAGMENT	Strain 1000x diluted	*Lichtheimia corymbifera*	18S sequencing	POSITIVE	30	NEGATIVE	-	NEGATIVE	-
HEPATIC ABCES	Sample	*Rhizomucor spp.*	26S sequencing	NEGATIVE	-	NEGATIVE	-	POSITIVE	33.6
TRANSBRONCHIAL BIOPSY *	Sample	*Lichtheimia*	PCR Besançon /CQE	NEGATIVE	-	NEGATIVE	-	NEGATIVE	-
SERUM	Sample	*Rhizomucor pusillus*	PCR Besançon /CQE	NEGATIVE	-	NEGATIVE	-	POSITIVE	37.6
SERUM	Sample	*NEGATIVE*	PCR Besançon /CQE	NEGATIVE	-	NEGATIVE	-	NEGATIVE	-
SERUM	Sample	*Rhizopus oryzae*	PCR Besançon /CQE	NEGATIVE	-	POSITIVE	35.1	NEGATIVE	-
SERUM	Sample	*Cunnighamella spp.*	PCR Besançon /CQE	NEGATIVE	-	NEGATIVE	-	NEGATIVE	-
SERUM *	Sample	*Lichtheimia corymbifera*	PCR Besançon /CQE	POSITIVE (1/2)	37	NEGATIVE	-	NEGATIVE	-
LAPAROTOMY FRAGMENT *	Sample	*Mucor circinelloides*	microscopy	POSITIVE	25.3	POSITIVE	22.4	POSITIVE	38.8 (1/2)
APPENDICE FRAGMENT *	Sample	*Mucor spp.*	microscopy	POSITIVE	33.9	POSITIVE	22.7	NEGATIVE	-

26S or 18S sequencing : a variable portion of the 26S or 18S rRNA gene was sequenced ; * discordance with Besançon result ; * the PCR allow to detect several Mucoales in contrast to the culture

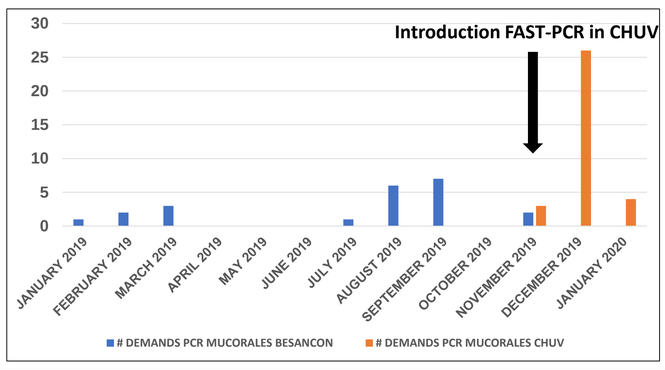
NEGATIVE SAMPLES :

SAMPLES	PCR LICHTEMIA	PCR MUCOR/RHIZOPUS	PCR RHIZOMUCOR
BAL	NEGATIVE	NEGATIVE	NEGATIVE
BIOPSY VENTRICULE	NEGATIVE	NEGATIVE	NEGATIVE
BAL	NEGATIVE	NEGATIVE	NEGATIVE
LIQUIDS	NEGATIVE	NEGATIVE	NEGATIVE
VERTEBRAL FRAGMENT	NEGATIVE	NEGATIVE	NEGATIVE
BAL	NEGATIVE	NEGATIVE	NEGATIVE
BAL	NEGATIVE	NEGATIVE	NEGATIVE
BAL	NEGATIVE	NEGATIVE	NEGATIVE
BAL	NEGATIVE	NEGATIVE	NEGATIVE
BRONCHOSCOPY	NEGATIVE	NEGATIVE	NEGATIVE

Lichteimia **could not or stochasticaly be detected for two samples with very low amount of material.**

We could guess that our PCR is slightly less sensitive than the Besançon one, however the FAST platform requires 5 times less starting material and twice less DNA volume.

V - Evolution of the Mucorales PCR demand:



Clearly the availability of the Mucorales PCR at the CHUV responds to a need of the clinicians with an increase demand since its introduction.

Conclusion

- This study allow us to introduce the detection of *Rhizopus, Mucor, Lichteimia*, and *Rhizomucor* on our Fast qPCR platform with a possible one-day result.
- The availability of Mucorales PCR onsite clearly correspond to a clinician need.

1- Millon *et al.,* Clin. Infect. Dis. (56) 2013. 2- Greub *et al*. Future Microbiology (11), 2016.