Detection and quantification by UDPS of microbial minority variants: Application for resistance mutations detection in *Aspergillus fumigatus*

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Introduction: Azoles are major agents for the treatment of aspergillosis but *Aspergillus fumigatus* resistance has appeared recently and increased in the last decade. The TR_{34}/L98H mutations in *cyp51a* are the most prevalent, but isolates with others substitutions were also found. **Wild type and resistant variants, could co-exist in various proportions in a respiratory sample from one patient**. Thus, to detect resistant variants, which may pre-exist as minority populations, a high sensitivity technology such as Ultra-Deep Pyro-Sequencing (UDPS) is required, especially to quantify these variants.

Our purpose was to **develop, validate and test a sensitive UDPS technique for the detection and quantification of *cyp51a* genetic variants variants of *A. fumigatus* isolates obtained from primary culture of respiratory samples.**

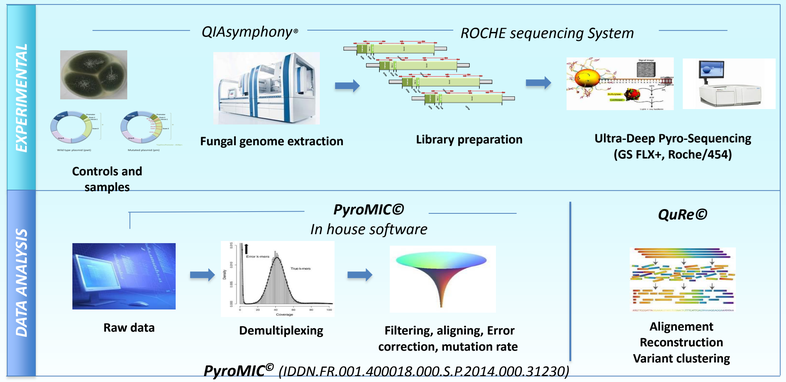
Materials and methods

Materials

- **Two control plasmids** :
-1 containing one wild type copy of *cyp51a* gene (pwt) with its promotor
1 containing different known-resistance mutations (pm)
were mixed in various proportions to reach 100%, 10%, 5%, 2%, 1%, 0% of pm to test sensitivity of UDPS technic.
- **32 primary cultures of respiratory samples** containing *A. fumigatus* were collected from 32 patients. A suspension of spores from the different colonies, obtained by swabing the entire surface of the tube, is used for the analysis.

Experimental methods

- Fungal DNA of each sample was extracted by **QIAsymphony©** and amplified by 5 different PCR along *cyp51a* and promotor.
- Sample's **libraries** were prepared from amplicons and sequenced by means of GS Junior or GS FLX+ sequencer (454/Roche) of according to Roche protocol.
- **Sanger sequencing**, using big Dye terminator kit on ABI 3130 sequencer were performed to confirm UDPS results.
- **Classical phenotypic tests** of azoles susceptibility was performed by Etest® from one colony of *A. fumigatus* isolated from primary culture to confirm UDPS results.
- **Screening phenotypic test** (plate with antifungals) of azoles resistance was performed from mix of *A. fumigatus* colonies isolated from primary culture.

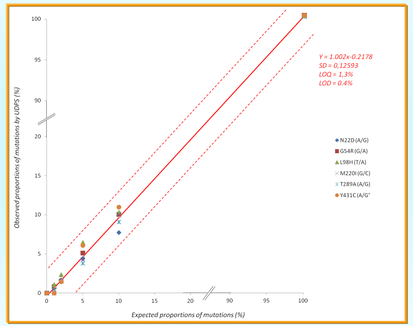


Data Analysis

Raw sequences were demultiplexed, filtered and analyzed using an **in-house software "PyroMIC©"**, to allow **quantification of mutations**. In parallel, from identic filtering sequences, QuRe © software was used to reconstruct *cyp51a* gene and analyzed population diversity.

Results of controls

Over 100,000 sequences of good quality (average length: 439 bp) were generated for controls and used for technical validation. **Data analysis was performed using PyroMIC© :** This software use a number of quality filters to eliminate unreliable sequences. Then variant calling is used to establish amino acid frequencies of different mutations or variants.
Results were used to establish **quantitativity of the method by linear regression (Figure 1)**.



Differences between expected and observed proportions of mutations were determined non significant (slope = 1.002). **Limit of detection (LOD)** and **limit of quantification (LOQ)** were calculated respectively at **0.4%** and **1.3%** for whole tested mutations.

Figure 1 : Linear regression (continuous line) and confidence interval (stippled line) between observed proportions of different tested mutations obtained by UDPS and expected results. Standard deviation on intercept (SD) were used to determine Limit of detection (LOD) and Limit of quantification (LOQ).

Results of samples

More than 400,000 sequences of good quality were generated for isolates obtained from respiratory samples (2625X coverage on average). Among these, **15.6 % (5/32) had a genetic variability** of *cyp51a* and for **6% (2/32), several variants co-exist. For one patient we have detected a resistant variant (29%) associated with the wild type (24%).** When different variants were found, conventional technics failed to detect resistance (Sanger sequencing and phenotypic tests) and could be discordant but results could be explained by UDPS **(Figure 2**).

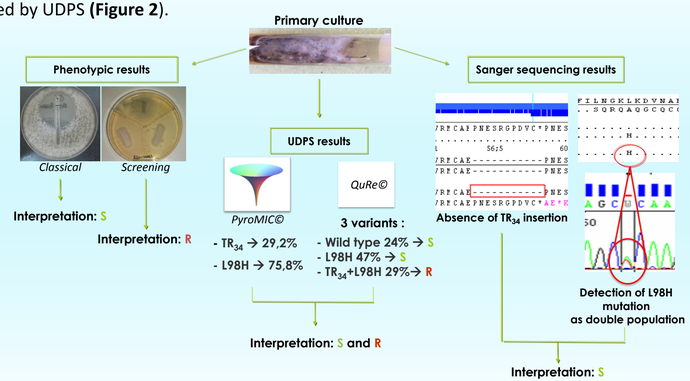


Figure 2: Phenotypic, genotypic and UDPS results for one sample containing wt and resistant variants. Because of the presence of different variants, classical and screening phenotypic results were discordant, classical method used in medical laboratory failed to detect resistance. Sanger sequencing results allowed detection of L98H as double population but failed to detect TR_{34} insertion which was found at 29% by UDPS. Finally, UDPS found 3 variants which could explained all results obtained by other methods. (S: Susceptible, R: Resistant)

Conclusion:

We have set-up an original and sensitive method of analysis of *cyp51a* diversity in *A. fumigatus*. This method allowed the detection of azole-resistance even as a small proportion of the whole diversity. **This technic could be used to explain differences observed with conventional technics used to detect resistance and thus may modify our laboratory practices.** Analysis of UDPS results allowed to detect *cyp51a* variants and showed that resistant variants could co-exist with wild type, it was also the only technic that give the proportion of mutation. The impact of this co-existence must be further explored because it could affect the success of treatment of patients.

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