Simple and rapid detection of TR34/L98H mutations in the *Cyp51A* gene of triazole-resistant *Aspergillus fumigatus* by tetra-primer ARMS-PCR technique

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Invasive aspergillosis (IA) is a life-threatening fungal infection with high morbidity and mortality in immunocompromised individual. The emergence of azole resistance is a serious problem and has been shown to be associated with a higher rate of treatment failure up to 90%. The majority of azole-resistant *A. fumigatus* isolates harboured TR34/L98H. Therefore, rapid detection of triazole-resistance associated point mutations is crucially important for the management of aspergillosis, appropriate treatment and epidemiologic studies. The tetra primer ARMS-PCR technique is efficient for SNP detection and can be used to search for polymorphisms associated with drug resistance. Herein, we describe an inexpensive, reliable and rapid simple ARMS-PCR assay using tetra-primers for rapid detection of TR34/L98H mutations in the *cyp51A* gene.

In this study, a panel of forty-five well-characterized clinical and environmental *A. fumigatus* strains, comprising azole-resistant (n = 20) and azole-susceptible (wild type; n = 25) strains were tested. Tetra-primer ARMS-PCR technique uses four primers, two external for detection of a 34 bp tandem repeat (in promoter region) and two internal for detection a point mutation (T→A) results in amino acid substitution in codon 98 (L98H) in the *Cyp51A* gene, in the same reaction, one primer to anneal to the non-mutated DNA and one primer to anneal to the mutated DNA. The primers employed in this study were designed using Primer 1 online. A portion (7μl) of PCR products was subjected for electrophoresis on 2% w/v agarose gels. Validation assay from tetra primer ARMS PCR result was done using DNA sequencing for all analyzes isolates.

Agarose gel of tetra-primer ARMS-PCR amplicons from *A. fumigatus* isolates.

The amplicon of 942 bp and 212 bp in lanes 1, 2, and 3 indicates the presence of TR34/L98H in triazole resistant *A. fumigatus* isolates, while the amplicon of 908 bp and 740 bp in lanes 5, 6, and 7 indicates absence of TR34 and L98H mutation (wild-type sequence) in triazole susceptible *A. fumigatus* isolates. Lane 4 is 100 bp DNA ladder.

Illustration of tetra-primer ARMS-PCR assay for detection of TR34/L98H mutation in the *Cyp51A* gene of triazole-resistant *A. fumigatus*.

- (1) a. Forward inner primer does not result in PCR product in wild-type sequence (T allele).
b. Reverse inner primer does not result in PCR product in TR34/L98H positive sequence (A allele).
- (2) a. The wild-type sequence (T allele) results in two products: 908 bp and 740 bp.
b. The TR34/L98H positive sequence (A allele) results in two products: 942 bp and 212 bp.
- (3) Lanes 1. 100 bp size marker;
Lane 2. The indicative amplicons of TR34/L98H positive sequence;
Lane 3. The indicative amplicons of wild-type sequence

The tetra-primer ARMS-PCR assay from reference *A. fumigatus* isolates containing wild-type sequence (no tandem repeat 34 and T allele), and L98H mutation at *cyp51A* (with tandem repeat 34 and A allele) yielded two (908 bp and 740 bp) and two (942 bp, 212 bp) DNA fragments, respectively. In this regard, the outer primers in the wild-type and TR34/L98H positive sequences resulted in 908 bp and 942 bp fragments, respectively. Also allele-specific inner primers yielded amplicons of 740 bp (indicator of T allele or wild-type) and 212 bp (indicator of A allele or L98H mutant), as expected. None of the *A. fumigatus* isolates lacking TR34/L98H mutations and other mutations yielded false-positive results by tetra primer ARMS PCR assays. The detection of TR34/L98H mutations in the *cyp51A* gene of all analyzed isolates by tetra primer ARMS PCR was confirmed with DNA sequencing.