UMM
UNIVERSITÄTSMEDIZIN
MANNHEIM

Medizinische Fakultät Mannheim
der Universität Heidelberg

Universitätsklinikum Mannheim

# Comparison of two molecular assays concerning detection and characterization of *Aspergillus fumigatus* azole resistance and *cyp51A* mutations in clinical isolates and primary clinical samples of immunocompromised patients

Birgit Spiess

Department of Hematology and Oncology
University Hospital Mannheim, University of Heidelberg, Germany

27th ECCMID Vienna, Austria 22 – 25 April 2017

ESCMID MANAGING INFECTIONS PROMOTING SCIENCE

# *Aspergillus* infections: clinical features, diagnosis and azole resistance

- Patients with long-term neutropenic phases after intensive chemotherapy (patients with acute leukemia under induction therapy or after allogenic stem cell transplantation) have a high risk of developing systemic fungal infections, primarily caused by *Aspergillus fumigatus*. The mortality rate due to the infection is high.

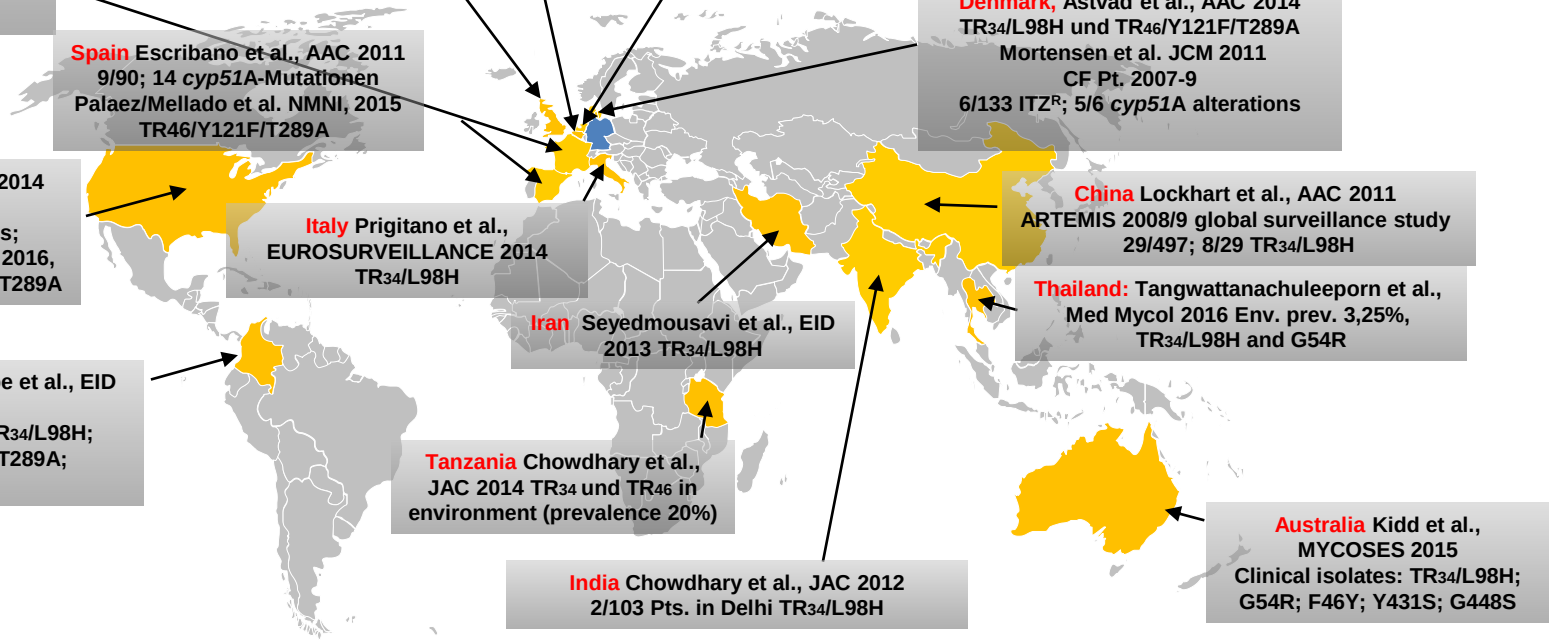
- In addition to improving the early diagnosis of invasive fungal infections, it is of increasing clinical relevance to detect antifungal resistance mechanisms in order to optimize the specific treatment.

- Prophylaxis in high-risk patients and therapy of proven/probable *Aspergillus* infections is performed with azoles (Voriconazole, Posaconazole, Isavuconazole).

- The formation of resistances against azoles in *Aspergillus fumigatus* can be carried out under azole therapy or as a result of environmental conditions (plant protection by azoles).

- Azole resistance mediating mutations and mutation combinations in the *cyp51A* gene (14α-sterol-demethylase) are currently the focus of attention worldwide.

# Published studies



**France**
Alaino et al. JAC 2011;
hematological pts;
Burgel et al., AAC 2012
CF Pts., 6/131; 5/6 *cyp51*A mutations;
Rocchi/Million et al., JCM 2014,
$TR_{34}$/L98H

**Belgium** Vermeulen et al., EURSURVEILLANCE 2012
$TR_{46}$/Y121F/T289A

**NL** Snelders/Verweij et al., PLOS ONE 2008,
$TR_{34}$/L98H; Van der Linden/Verweij et al., CID 2013, $TR_{46}$/Y121F/T289A
Kuijper et al., EID 2011
2007-9: 2,062 Isolate (5,3% $ITZ^R$)
Van Paassen et al., 2016
10/38 proven IA ICU Pt.: 2010-2013
$TR_{34}$/L98H; $TR_{46}$/Y121F/T289A

**UK** Howard et al., JAAC 2006, EID 2009, multi-azole-resistance
Denning et al., AAC 1997, CID 2011,
$TR_{34}$/L98H

**Spain** Escribano et al., AAC 2011
9/90; 14 *cyp51*A-Mutationen
Palaez/Mellado et al. NMNI, 2015
TR46/Y121F/T289A

**Denmark,** Astvad et al., AAC 2014
$TR_{34}$/L98H und $TR_{46}$/Y121F/T289A
Mortensen et al. JCM 2011
CF Pt. 2007-9
6/133 $ITZ^R$; 5/6 *cyp51*A alterations

**USA** Pham et al., EID 2014
No $TR_{46}$ or TR34
>1000 tested isolates;
Wiederhold et al., JCM 2016,
$TR_{34}$/L98H, $TR_{46}$/Y121F/T289A

**Italy** Prigitano et al.,
EUROSURVEILLANCE 2014
$TR_{34}$/L98H

**China** Lockhart et al., AAC 2011
ARTEMIS 2008/9 global surveillance study
29/497; 8/29 $TR_{34}$/L98H

**Thailand:** Tangwattanachuleeporn et al.,
Med Mycol 2016 Env. prev. 3,25%,
$TR_{34}$/L98H and G54R

**Iran** Seyedmousavi et al., EID 2013 $TR_{34}$/L98H

**Columbia** Le Pape et al., EID 2016
Flower field: $TR_{34}$/L98H;
$TR_{46}$/Y121F/T289A;
$TR_{53}$

**Tanzania** Chowdhary et al.,
JAC 2014 $TR_{34}$ und $TR_{46}$ in environment (prevalence 20%)

**India** Chowdhary et al., JAC 2012
2/103 Pts. in Delhi $TR_{34}$/L98H

**Australia** Kidd et al.,
MYCOSES 2015
Clinical isolates: $TR_{34}$/L98H;
G54R; F46Y; Y431S; G448S

By courtesy of Dr. O. Bader, Göttingen, Germany (modified).

## Development of Novel PCR Assays To Detect Azole Resistance-Mediating Mutations of the *Aspergillus fumigatus cyp51A* Gene in Primary Clinical Samples from Neutropenic Patients

Birgit Spiess,[a] Wolfgang Seifarth,[a] Natalia Merker,[a] Susan J. Howard,[b] Mark Reinwald,[a] Anne Dietz,[c] Wolf-Karsten Hofmann,[a] and Dieter Buchheidt[a]

Antimicrobial Agents and Chemotherapy

## First Reported Case of Azole-Resistant Aspergillus fumigatus Due to the TR/L98H Mutation in Germany

P.-M. Rath, D. Buchheidt, B. Spiess, E. Arfanis, J. Buer and J. Steinmann

*Antimicrob. Agents Chemother.* 2012, 56(11):6060. DOI:

RAPID COMMUNICATIONS

## Azole-resistant invasive aspergillosis in a patient with acute myeloid leukaemia in Germany

A Hamprecht (axel.hamprecht@uk-koeln.de)[1], D Buchheidt[2], J J Vehreschild[3], O A Cornely[3,4], B Spiess[1], G Plum[1], T V Halbsguth[1], N Kutsch[1], D Stippel[1], P Kahl[1], T Persigehl[1], A Steinbach[1], B Bos[1], M Haltek[1], M J Vehreschild[1]
1. Institute for Medical Microbiology, Immunology and Hygiene, University of Cologne, Cologne, Germany
2. Third Department of Internal Medicine, Haematology and Oncology, Mannheim University Hospital, University of Heidelberg

**April 2017**:
**15 hematological pat.** with detected
*A. fumigatus* azole resistance.

## Incidence of *Cyp51 A* Key Mutations in *Aspergillus fumigatus*—A Study on Primary Clinical Samples of Immunocompromised Patients in the Period of 1995–2013

Birgit Spiess[1,*], Patricia Postina[1,], Mark Reinwald[1], Oliver A. Cornely[2], Axel Hamprecht[3], Martin Hoenigl[4], Cornelia Lass-Flörl[5], Peter-Michael Rath[6], Jörg Steinmann[6], Thomas Miethke[7], Melchior Lauten[8], Silke Will[1], Natalia Merker[1], Wolf-Karsten Hofmann[1], Dieter Buchheidt[1]

## *cyp51A*-Based Mechanisms of *Aspergillus fumigatus* Azole Drug Resistance Present in Clinical Samples from Germany

Oliver Bader,[a] Michael Weig,[a] Utz Reichard,[a] Raimond Lugert,[a] Martin Kuhns,[a] Martin Christner,[b] Jürgen Held,[c] Silke Peter,[d] Ulrike Schumacher,[d]* Dieter Buchheidt,[e] Kathrin Tintelnot,[f] Uwe Groß,[a] MykoLabNet-D Partners

## Emergence of azole-resistant invasive aspergillosis in HSCT recipients in Germany

J. Steinmann[1]*†, A. Hamprecht[2]†, M. J. G. T. Vehreschild[3,4], O. A. Cornely[3–5], D. Buchheidt[6], B. Spiess[6], M. Koldehoff[7], J. Buer[1], J. F. Meis[8,9] and P.-M. Rath[1]

Detection of a novel *cyp51A* $TR_{46}$/Y121F/M172I/T289A-allele in *A. fumigatus* in a patient with acute myeloid leukemia.
Susann Rößler, Oliver Bader, Friedrich Stölzel, Ulrich Sommer, Birgit Spiess, Stephan Geibel, Dieter Buchheidt, Uwe Groß, Gustavo Baretton, Enno Jacobs
Submitted to …

# Most frequent *cyp51A* mutations und mutation combinations

***cyp51A* promoter region**: Tandem Repeats: **TR34, TR46, …**

**Mutation combinations**:

**TR34/L98H**

Vermeulen/Lagrou , Belgium, Euro Surveill. 2012
Van der Linden/Verweij, Netherlands, CID 2013
Steinmann/Rath, Germany, JAC 2015

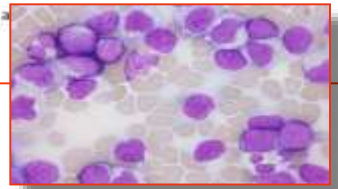
**TR46/Y121F/T289A**

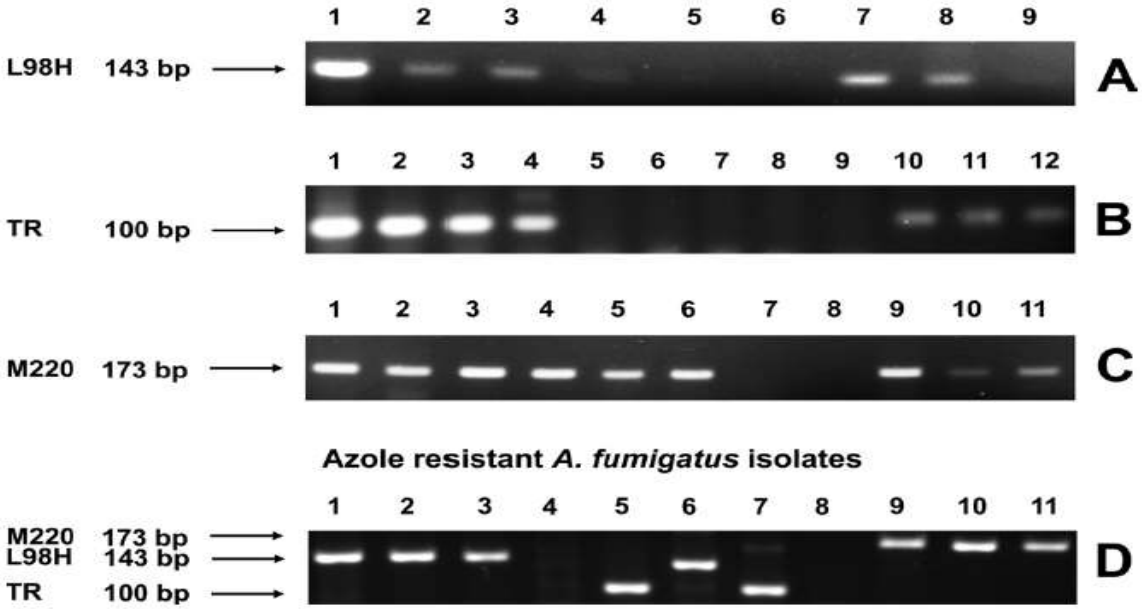
Van Ingen/Verweij, Netherlands, JCM 2015

- **high-level triazole resistance**

# Development of Novel PCR Assays To Detect Azole Resistance-Mediating Mutations of the *Aspergillus fumigatus cyp51A* Gene in Primary Clinical Samples from Neutropenic Patients

Birgit Spiess,[a] Wolfgang Seifarth,[a] Natalia Merker,[a] Susan J. Howard,[b] Mark Reinwald,[a] Anne Dietz,[c] Wolf-Karsten Hofmann,[a] and Dieter Buchheidt[a]






July 2012 Volume 56 Number 7 Antimicrobial Agents and Chemotherapy p. 3905–3910

**Clinical sample** (BAL, biopsy, liquor cerebrospinalis**)**

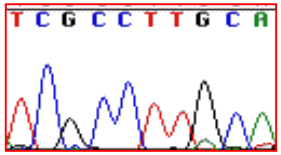
Diagnostic nested *Aspergillus* PCR

(Skladny et al. 1999: target gene 18S rRNA;
sensitivity 10 fg gen. *Aspergillus* DNA; 3-5 CFU/ml)

**(positivity)**

TR46 PCR

TR34, M220, L98H, Y121F, T289A




Sanger DNA sequencing

Diagnostic findings

# Mannheim *Aspergillus* azole resistance PCRs (ARAf-PCRs)

| **Spiess et al. 2012: AAC (26):3905-3910** | | | |
|---|---|---|---|
| **Mutation** | Fragment length | PCR procedure | Sensitivity |
| **L98H** | 143 bp | One-Step | 6 pg |
| **TR34** | 235 bp<br>100 bp | Two-Step | 600 fg |
| **M220** | 173 bp | One-Step | 4 pg |
| **Spiess et al. 2014: PLOS ONE (9):e103113** | | | |
| **Mutation** | Fragment length | PCR procedure | Sensitivity |
| **L98H** | 143 bp | One-Step | 300 fg |
| **TR46** | 213 bp<br>103 bp | Two-Step | 300 fg |
| **Postina/Spiess et al.: unpublished** | | | |
| **Mutation** | Fragment length | PCR procedure | Sensitivity |
| **Y121F** | 101 bp | One-Step | 300 fg |
| **T289A** | 133 bp | One-Step | 300 fg |

# Pathonostics AsperGenius® PCR system

**LightCycler 480 (Roche)**
Rotor-Gene 6000 (Corbett)
Rotor-Gene (Qiagen)

*Table 6. Filter settings for optimal detection of AsperGenius® probes*

| Species multiplex | Resistance multiplex | Detection Channel | Rotor-Gene (nm) | | LC480 II (nm) | |
|---|---|---|---|---|---|---|
| | | | Source | Detector | Source | Detector |
| *A. fumigatus* | L98H | green | 470 | 510 | 465 | 510 |
| *A. terreus* | TR34 | yellow | 530 | 555 | 533 | 580 |
| A. species | T289A | orange | 585 | 610 | 533 | 610 |
| IC | Y121F | red | 625 | 660 | 618 | 660 |

WT mutant

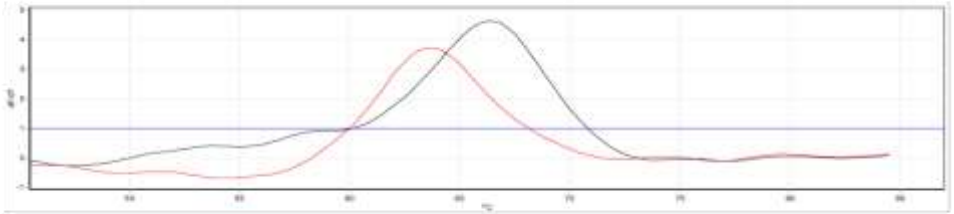


**Figure 11. TR34 melting peaks.** Red melting peak indicates the wildtype ($T_m$ = 64.5), black indicates the TR34 mutant ($T_m$ = 66.8)

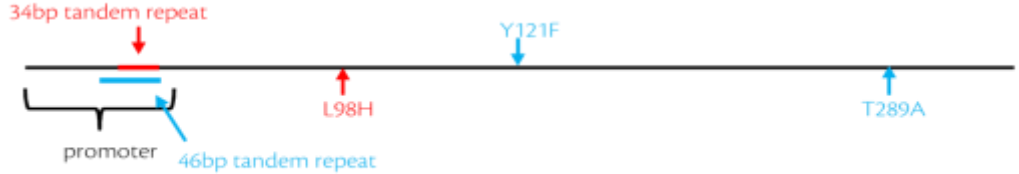



Figure 1. Schematic overview of the CYP51A gene of *A.fumigatus*

# AsperGenius® PCR system: publications

JCM

Analytical and Clinical Evaluation of the PathoNostics AsperGenius Assay for Detection of Invasive Aspergillosis and Resistance to Azole Antifungal Drugs during Testing of Serum Samples

P. Lewis White,[a] Raquel B. Posso,[b] Rosemary A. Barnes[b]

Public Health Wales Microbiology Cardiff, Cardiff, United Kingdom[a]; Infection, Immunity and Biochemistry, School of Medicine, Cardiff University, Cardiff, United Kingdom[b]

JCM 2015; 53 (7):2115-2121

JCM

Validation of a New *Aspergillus* Real-Time PCR Assay for Direct Detection of *Aspergillus* and Azole Resistance of *Aspergillus fumigatus* on Bronchoalveolar Lavage Fluid

Ga-Lai M. Chong,[a] Wendy W. J. van de Sande,[b] Gijs J. H. Dingemans,[c] Giel R. Gaajetaan,[c] Alieke G. Vonk,[b] Marie-Pierre Hayette,[d] Dennis W. E. van Tegelen,[e] Guus F. M. Simons,[e] Bart J. A. Rijnders[a]

Department of Internal Medicine, Infectious Diseases, Erasmus Medical Center, Rotterdam, the Netherlands[a]; Medical Microbiology and Infectious Diseases, Erasmus Medical Center, Rotterdam, the Netherlands[b]; PathoNostics B.V., Maastricht, the Netherlands[c]; Department of Clinical Microbiology, University Hospital of Liège (CHU), Liège, Belgium[d]

JCM 2015; 53 (3):868-874

Journal of Antimicrobial Chemotherapy Advance Access published August 15, 2016

*J Antimicrob Chemother*
doi:10.1093/jac/dkw323

Journal of Antimicrobial Chemotherapy

PCR-based detection of *Aspergillus fumigatus Cyp51A* mutations on bronchoalveolar lavage: a multicentre validation of the AsperGenius assay® in 201 patients with haematological disease suspected for invasive aspergillosis

G. M. Chong[1]*, M. T. van der Beek[2], P. A. von dem Borne[3], J. Boelens[4], E. Steel[5], G. A. Kampinga[6], L. F. R. Span[7], K. Lagrou[8], J. A. Maertens[9], G. J. H. Dingemans[10], G. R. Gaajetaan[10], D. W. E. van Tegelen[10], J. J. Cornelissen[11], A. G. Vonk[12] and B. J. A. Rijnders[1]

JAC 2016; doi:10.1093

# Experimental design of comparative analysis

- Comparison of the six Mannheim ARAf PCR assays (plus DNA sequence analysis) with the commercial AsperGenius® test system for the detection of the azole resistance mutations TR34/L98H; TR46/Y121F/T289A directly from clinical samples.

- No PCR-based diagnostic *Aspergillus* DNA detection with the AsperGenius® system.

- Retrospective analysis of assured DNA aliquots, which had a positive detection of *Aspergillus* DNA in our diagnostic nested *Aspergillus* PCR (Skladny et al., 1999).

- DNA processing of the clinical samples by enzymatic cleavage, mechanical comminution (biopsates) and phenol-chloroform purification (protocol Mannheim).

# Patients and clinical samples

**Patients:**

number: n=56

**Main diseases:**

ALL: 11

AML:10

CLL: 3

T-PLL: 1

MDS: 2

NHL: 14

Hodgkin lymphoma: 1

solid tumor: 6

autoimmune neutropenia: 1

immunosuppression (NOS): 7

**Clinical samples:**

BAL: 22

biopsies: 15

liquor cerebrospinalis: 15

pleura effusion: 4

**EORTC criteria (IA):**

proven: 12

probable: 15

possible: 17

no IA: 12

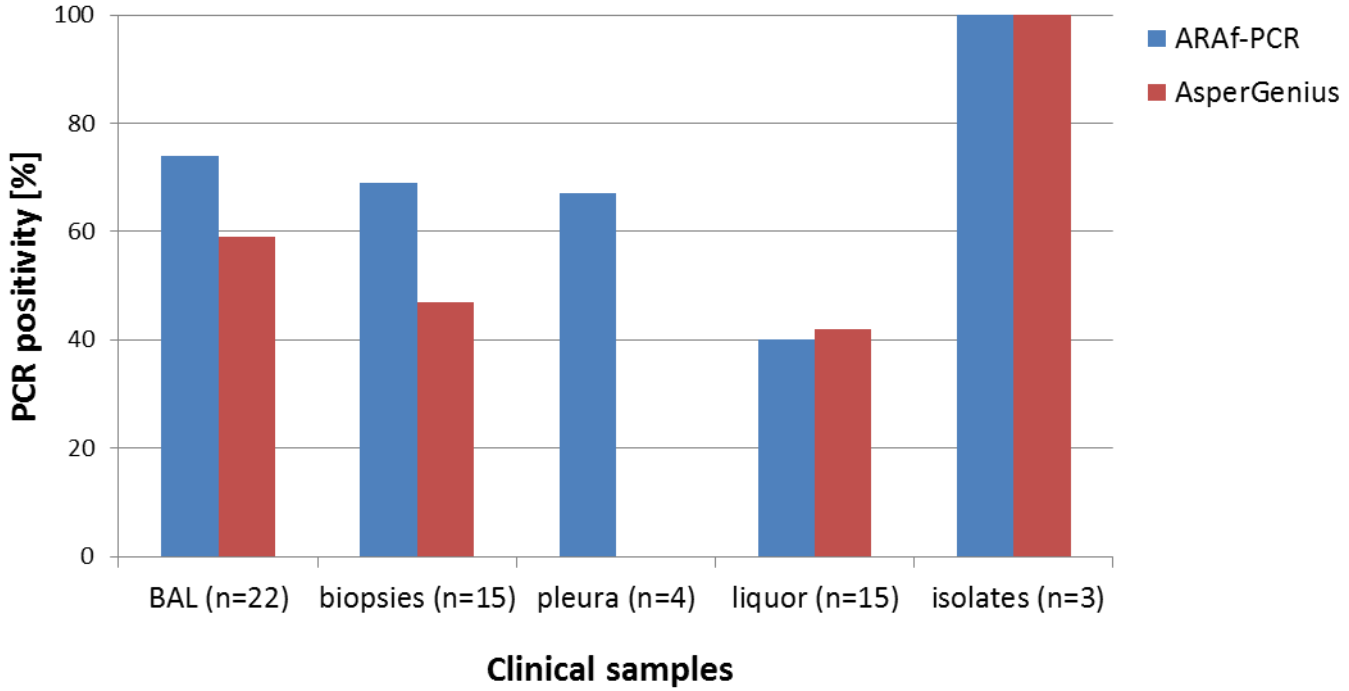
# Proportion of positive PCR results in comparison - BAL

| | | Mannheim ARAf-PCR | | AsperGenius® | |
|---|---|---|---|---|---|
| | | PCR+ | Mutation+ | PCR+ | Mutation+ |
| BAL | TR34 | 82 % (18/22) | 1 | 64 % (14/22) | 1 |
| | L98H | 91 % (20/22) | 1 | 31 % (7/22) | 1 |
| | TR46 | 77 % (17/22) | 0 | - | - |
| | Y121F | 68 % (15/22) | 0 | 72 % (16/22) | 0 |
| | T289A | 59 % (13/22) | 0 | 68 % (15/22) | 0 |
| | M220 | 68 % (15/22) | 0 | - | - |
| | **Total** | **74 % (98/132)** | **2** | **59 % (52/88)** | **2** |

# Proportion of positive PCR results in comparison - biopsies

| | | Mannheim ARAf-PCR | | AsperGenius® | |
|---|---|---|---|---|---|
| | | PCR+ | Mutation+ | PCR+ | Mutation+ |
| Biopsies | TR34 | 53 % (08/15) | 1 | 33 % (05/15) | 0 |
| | L98H | 67 % (10/15) | 3 | 33 % (05/15) | 0 |
| | TR46 | 60 % (09/15) | 1 | - | - |
| | Y121F | 80 % (12/15) | 1 | 60 % (09/15) | 1 |
| | T289A | 80 % (12/15) | 1 | 60 % (09/15) | 1 |
| | M220 | 73 % (11/15) | 0 | - | - |
| | **Total** | **69 % (62/90)** | **7** | **47 % (28/60)** | **2** |

# Proportion of positive PCR results in comparison



# Mutation-positive samples in comparison

| Clinical samples and isolates | Mannheim ARAf-PCRs plus sequencing | | | | | | AsperGenius® | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | TR34 | L98H | TR46 | Y121F | T289A | M220 | TR34 | L98H | Y121F | T289A |
| BAL: (AML) (Hamprecht et al., 2012) | + | + | - | - | - | - | + | + | - | - |
| Lung biopsy: (AML) | - | + | - | - | - | - | - * | - * | - * | - * |
| Brain biopsy: (ALL) | + | + | - | - | - | - | - * | - * | - | - |
| Lung biopsy: (Osteosarcoma) | - * | + | - * | - | - * | - | - * | - * | - * | - * |
| Lung biopsy (env273TR46+): (AML) | - | - | + | + | + | - | - * | - * | + | + |
| Isolate of biopsy (env273TR46+): (AML) (PC for TR46/Y121F/T289A) | - | - | + | + | + | - | - | - | + | + |
| Isolate of BAL: (AML) (Hamprecht et al. 2012) | + | + | - | - | - | - | + | + | - | - |
| Isolate (IMMi 2107) (TR46+ isolate): (PC for TR46/Y121F/T289A) | - | - | + | + | + | - | - | - | + | + |

* DNA not amplified; PC = positive control

# Summary

- The molecular detection of azole resistance mutations plays a major role in resistance diagnostics due to mostly negative *Aspergillus* cultures.

- Detection of the *cyp51A* mutations TR34/L98H; TR46/Y121F/T289A and M220 by Mannheim ARAf PCRs plus sequencing (**6 gene alterations**).

- Detection of the *cyp51A* mutations TR34/L98H and Y121F/T289A by the AsperGenius® Pathonostics assay system (**4 gene alterations**).

- Detection of a total of 17 mutations with the Mannheim ARAf-PCRs (4 x biopsy, 1 x BAL, 3 x isolate) and 10 mutations with the AsperGenius® system (1 x biopsy, 1 x BAL, 3 x isolate).

- The major advantage of the AsperGenius® system is the time-saving against sequence-based analysis.

UMM
UNIVERSITÄTSMEDIZIN
MANNHEIM

Medizinische Fakultät Mannheim
der Universität Heidelberg
Universitätsklinikum Mannheim

**Department of Hematology and Oncology (Director: Prof. Dr. W.-K. Hofmann)**

**Scientific laboratory**

**Working group „Molecular diagnostics of invasive fungal infections“**


Birgit Spiess

Julian Skladny

Patricia Postina

Natalia Merker

Tobias Boch

Dieter Buchheidt

# Cooperating centers

| | |
|---|---|
| University Hospital Cologne | Prof. Dr. O. A. Cornely, PD Dr. A. Hamprecht |
| University Hospital Schleswig-Holstein<br>Campus Lübeck, Lübeck | PD Dr. M. Lauten |
| Ruhr-University Bochum, Clinical Center Herne | Dr. B. Schultheis |
| University Hospital Essen,<br>Clinic for bone marrow transplantation | Dr. T. Liebregts |
| University Hospital Münster,<br>Dept. päd. Hematology and Oncology | Prof. Dr. A. Groll |
| University Hospital Essen,<br>Institut for Medical Microbiology | Prof. Dr. P.-M. Rath, PD Dr. J. Steinmann |
| University Hospital Mannheim;<br>Institut für University Hospital and Hygiene | Prof. Dr. T. Miethke, A. Dietz |
| University Hospital Göttingen,<br>Institut for Medical Microbiology | Dr. O. Bader, Prof. Dr. U. Groß |
| Friedrich-Schiller-University Jena,<br>Reference Center für invasive fungal infections | Prof. Dr. O. Kurzai |