Aspergillus Speciation in the 21st Century -

Implications for Laboratory and Clinical Practice

Recommendations for routine daily practice

WORKSHOP. 6TH AAA. Madrid

Manuel Cuenca-Estrella

Conflict of interest disclosure

- In the past 5 years, M.C.E. has received grant support from **Astellas Pharma, bioMerieux, Gilead Sciences, Merck Sharp and Dohme, Pfizer, Schering Plough, Soria Melguizo SA**, **Ferrer International**
- He has been an advisor/consultant to the **Panamerican Health Organization, Astellas Pharma, Gilead Sciences, Merck Sharp and Dohme, Pfizer, and Schering Plough**.
- He has been paid for talks on behalf of **Gilead Sciences, Merck Sharp and Dohme, Pfizer, Astellas Pharma and Schering Plough**.

Aspergillus spp.

Aspergillus **spp. are the most frequent moulds in the environment**

They play a significant role in decay of organic matter

1-100 conidia/m^3 of air

We inhale several hundreds a day

Aspergillus fumigatus

Epidemiology of moulds

	Hematological patients			SOT
	Neofytos CID'09	Pagano CID'07	Marr CID'02	Husain CID'03
Aspergillus	80%	94.5%	77.3%	69.8%
Zygomycetes	9.7%	1.1%	8.6%	5.6%
Fusarium	2.2%	3.2%	9.2%	3.7%
Scedosporium	---	1.1%	2.9%	5.6%
Other	9.2%	---	1.78%	15%

Prospective Surveillance for Invasive Fungal Infections in Hematopoietic Stem Cell Transplant Recipients, 2001–2006: Overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database

Clinical Infectious Diseases 2010;50:000–000

Invasive Fungal Infections among Organ Transplant Recipients: Results of the Transplant-Associated Infection Surveillance Network (TRANSNET)

Clinical Infectious Diseases 2010;50:000–000

	Haematological	SOT
A. fumigatus	44%	60%
A. terreus	5%	4%
A. niger	9%	6%
A. flavus	7%	7%
Unspecified Aspergillus	26%	7%
Zygomycetes	8%	2%
Fusarium	3%	---
Unspecified moulds	6%	2%

Results from Spain. FILPOP. Population-based survey. 325 isolates

Aspergillus spp. (*sensu stricto*)	N	%
TOTAL	277	85% (4 cases per 100,000 pop)
A. fumigatus	156	48%
A. flavus	26	8%
A. terreus	26	8%
A. tubingensis (section *Nigri*)	22	6.8%
A. niger	21	6.5%
A. nidulans	8	2.5%

Aspergillus cryptic species

11% cryptic species

15% cryptic species

Table 1. *Aspergillus* speci idemiological surveys from Spain and the U. S.[22]

Species	Section	Transnet N isolates	Transnet %	FILPOP N isolates	FILPOP %
A. fumigatus	Fumigati	139	63.8	156	56.1
A. lentulus	Fumigati	4	1.8	3	1.1
A. udagawae	Fumigati	3	1.4	0	0.0
N. peudofischeri	Fumigati	1	0.5	1	0.4
A. viridinutans	Fumigati	0	0.0	1	0.4
A. fumigatiafinis	Fumigati	0	0.0	1	0.4
A. flavus	Flavi	29	13.3	27	9.7
A. alliaceus	Flavi	0	0.0	3	1.1
A. terreus	Terrei	11	5.0	26	9.4
A. carneus	Terrei	0	0.0	1	0.4
A. tubingensis	Nigri	6	2.8	22	7.9
A. niger	Nigri	13	6.0	21	7.6
A. calidoustus	Usti	6	2.8	4	1.4
A. insuetus	Usti	0	0.0	1	0.4
A. keveii	Usti	0	0.0	1	0.4
A. sydowii	Versicolores	2	0.9	1	0.4
A. versicolor	Versicolores	3	1.4	0	0.0
E. quadrilineata	Nidulantes	1	0.5	0	0.0
A. nidulans	Nidulantes	0	0.0	8	2.9
A. westerdijkiae	Circumdati	0	0.0	1	0.4
Total		218	100	278	100

Alastruey-Izquierdo et al. ANYAS 2012

Cryptic Species

	n	AMB	ICZ	VCZ	PCZ	CPF	MCF	ANF
A. lentulus	26				**0.23**	1.6	0.1	0.1
N. hiratsukae	9	1.7	0.9	1.1	0.16	0.11	0.03	0.03
N. pseudofischerii	6	0.25			**0.22**	0.86	0.03	0.03
A. fumigatiaffiinis	6					0.22	0,03	0,03
N. udagawae	5	**2**	0.6		**0.25**	0.3	0.03	0.03
A. viridinutans	3	0,7			**0,25**	5,66	0,06	0,09
A. tubingensis	22	0.11	0.42	0.76	0.09	0.3	0.05	0.03
A. calidoustus	19	0.9				0.5	0.04	0.04
A. insuetus	2	0.7				5.6	1.4	0.9
A. keveii	1	0,25				16	16	16
A. alliaceus	30		0.2	0.5	0.11	12.15	3.8	1.9

By Alastruey-Izquierdo

The management of febrile neutropenia in the posaconazole era: a new challenge?

Livio Pagano,[1] Morena Caira[1] and Manuel Cuenca-Estrella[2]

[1]Istituto di Ematologia, Università Cattolica del S.Cuore, Rome, Italy; [2]Servicio de Micología, Centro Nacional de Microbiolo Salud Carlos III, Majadahonda, Madrid, Spain

E-mail: lpagano@rm.unicatt.it *doi:10.3324/haematol.2012.062166*

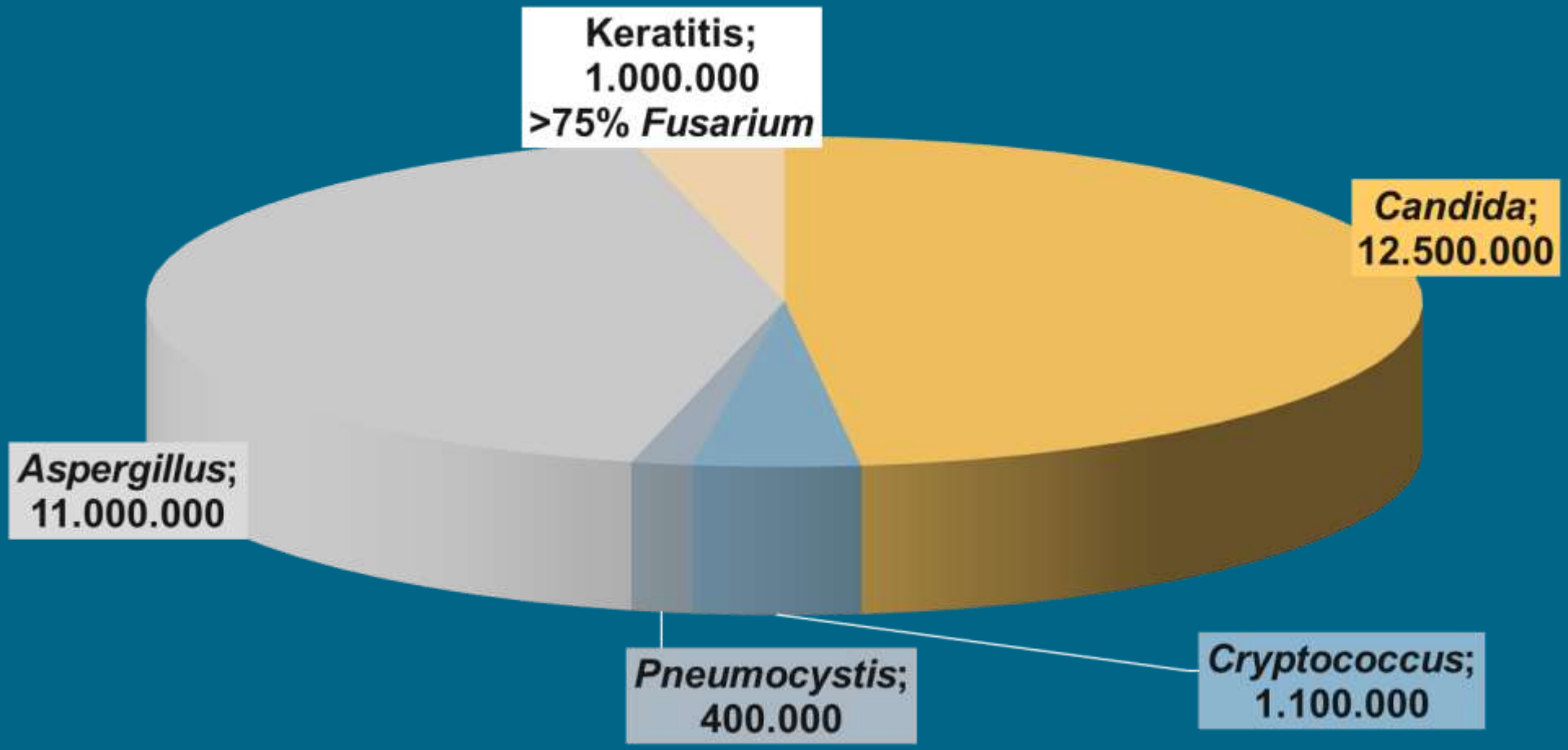
Table 1. Incidence of proven/probable invasive fungal diseases in acute myeloid leukemia after posaconazole prophylaxis: data from different types of study.

References	Years	Type of study	N. pts	N. proven/probable breakthrough IFDs	Incidence %
RCT					
Cornelly *et al.*,[8]	2002-05	RCT	304	7	2%
"Real life" studies					
Michallet *et al.*[19]	2007-08	Pros	55	2	3.6%
Candoni *et al.*[15]	2009-10	Retro	55	2	4%
Lerolle *et al.*[18]	2007-10	Retro	209	8	3.8%
Egerer *et al.*[16]	2007-09	Retro	76*	1	1.3%
Vehreschild *et al.*[20]	2006-08	Retro	77	3	3.9%
Hahn *et al.*[17]	2007-08	Retro	21	1	5%
Busca *et al.*[14]	2009-10	Retro	61	0	0
Ananda-Rajah *et al.*[13]	2006-10	Retro	68	0	0

*RCT: randomized clinical trial; Retro: retrospective study; Pros: prospective study; IFDs: invasive fungal diseases. * number of chemotherapy courses.*

Relevant Mycoses. Annual incidence

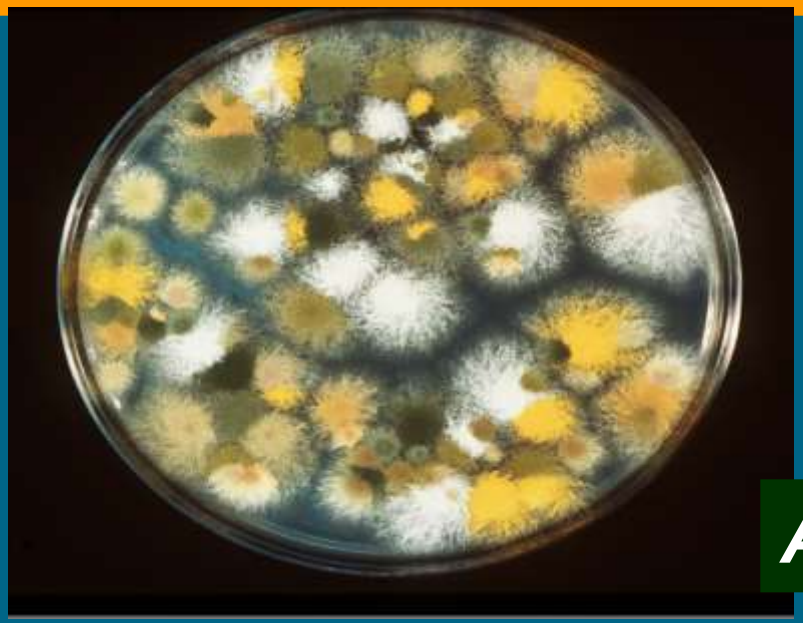
According to LIFE and GAFFI (www.life-worldwide.org)



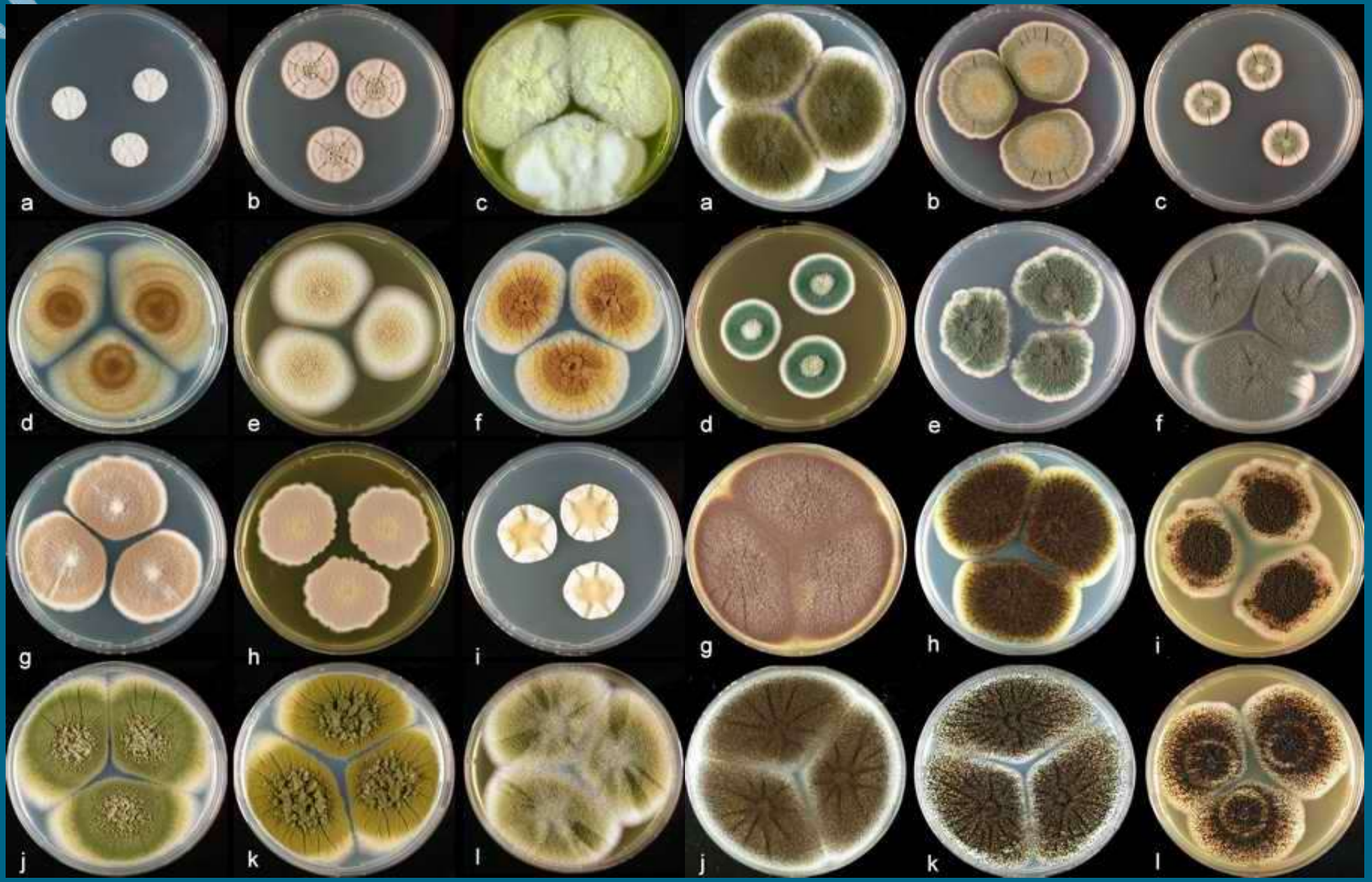
Aspergillus spp.

Aspergillus **spp. are (at least) 175 species, 18 complexes or sections (*perpetual motion*)**

10-15 sexual forms identified



Aspergillus **spp.**



Klich MA. Identification of common *Aspergillus* species (2002). CBS.

Unusual or atypical isolates

Poorly sporulating
A. fumigatus

Neosartorya fischeri with
masses of cleistothecia

Reasons for molecular identification in Medical Mycology

- Morphology is not enough
- Changing landscape of epidemiology (sibling and cryptic species)
- Species-specific differences in antifungal susceptibilities
- Quick lab answer (automated); better patient's outcome?

Consensus Molecular Taxonomy

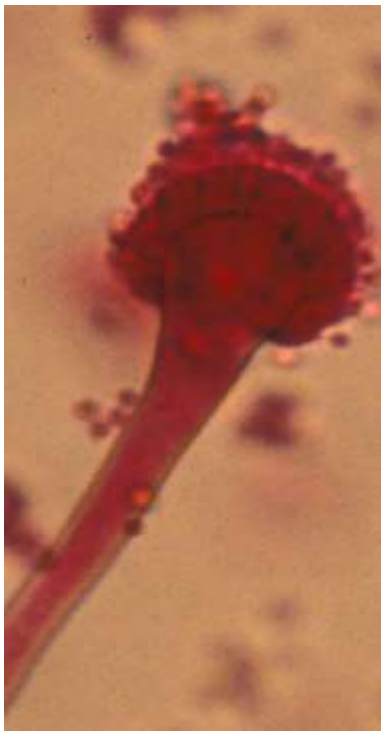
JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2009, p. 877–884 Vol. 47, No. 4
0095-1137/09/$08.00+0 doi:10.1128/JCM.01685-08

GUEST COMMENTARY

Sequence-Based Identification of *Aspergillus*, *Fusarium*, and *Mucorales* Species in the Clinical Mycology Laboratory: Where Are We and Where Should We Go from Here?[▽]

S. A. Balajee,[1]* A. M. Borman,[2] M. E. Brandt,[1] J. Cano,[3] M. Cuenca-Estrella,[4] E. Dannaoui,[5] J. Guarro,[3] G. Haase,[6] C. C. Kibbler,[7] W. Meyer,[8] K. O'Donnell,[9] C. A. Petti,[10] J. L. Rodriguez-Tudela,[4] D. Sutton,[11] A. Velegraki,[12] and B. L. Wickes[13]

Molecular id by means of sequencing β-tubuline of *A. fumigatus* species complex



Data obtained from Mycology Reference Laboratory. Spain

Culture of clinical samples

Population	Intention	Intervention	SoR	QoE	Reference	Comment
Any	Primary isolation from deep sites (biopsies, blood, CSF…)	Culture on SDA, BHI agar, PDA or PFA at 30ºC and 37ºC for 72 h	A	III	Cuenca-Estrella JAC 2011 Richardson HM 2000	Blood inhibits conidation BHI can help to recover some isolates Isolation of several colonies or isolation of the same fungus from a repeat specimen enhance significance
Any	Primary isolation from non-sterile samples (sputum, respiratory aspirates, skin…)	Culture on SDA, BHI agar, PDA or PFA with gentamicin PLUS chloramphenicol at 30ºC and 37ºC for 72 h	A	III	Cuenca-Estrella JAC 2011 Richardson HM 2000	Quantitative cultures are not useful and as above
Any	Primary isolation from BAL	As above	A	III	Cuenca-Estrella JAC 2011 Richardson HM 2000	Quantitative cultures are not discriminative for infection/colonization and as above

Morphology in primary cultures and subcultures

Population	Intention	Intervention	SoR	QoE	Reference	Comment
Any	Identification of species complex	Macroscopic and microscopic examination from primary cultures	A	III	Klich. Identification of Common *Aspergillus* Species. 2002 Cuenca-Estrella JAC 2011	Colony color Conidium size, shape and septation. Color of conidia and conidiophore and conidiogenesis (tease or tape mounts are preferred) Expertise needed for interpretation
Any	Identification of species complex	Culture on identification media at 25-30ºC and 37ºC (2% MEA and Czapek-Dox agar) and microscopic examination	A	III	As above	As above
Any	Identification of species complex	Culture at 45ºC	B	III	As above	Presumptive ID of *A. fumigatus* complex

Population/Test: Positive culture - MALDI-TOF

Population	Intention	Intervention	So R	Qo E	Reference	Comment
Any	To establish epidemiological knowledge and to guide treatment	MALDI-TOF MS identification	B	Ilu	Alanio 2011 Bille 2012 De Carolis 2012 Lau 2013	In house database

Positive culture. Molecular ID

Population	Intention	Intervention	SoR	QoE	Reference	Comment
Any	Identification at species level	Sequencing of ITS and beta-tubulin areas	B	III	Balajee JCM 2009 Samson. Studies in Mycology 59: *Aspergillus* systematics in the genomic era. 2007	Essential investigation in some cases Reference laboratory and databases can be needed (Mycobank at CBS)

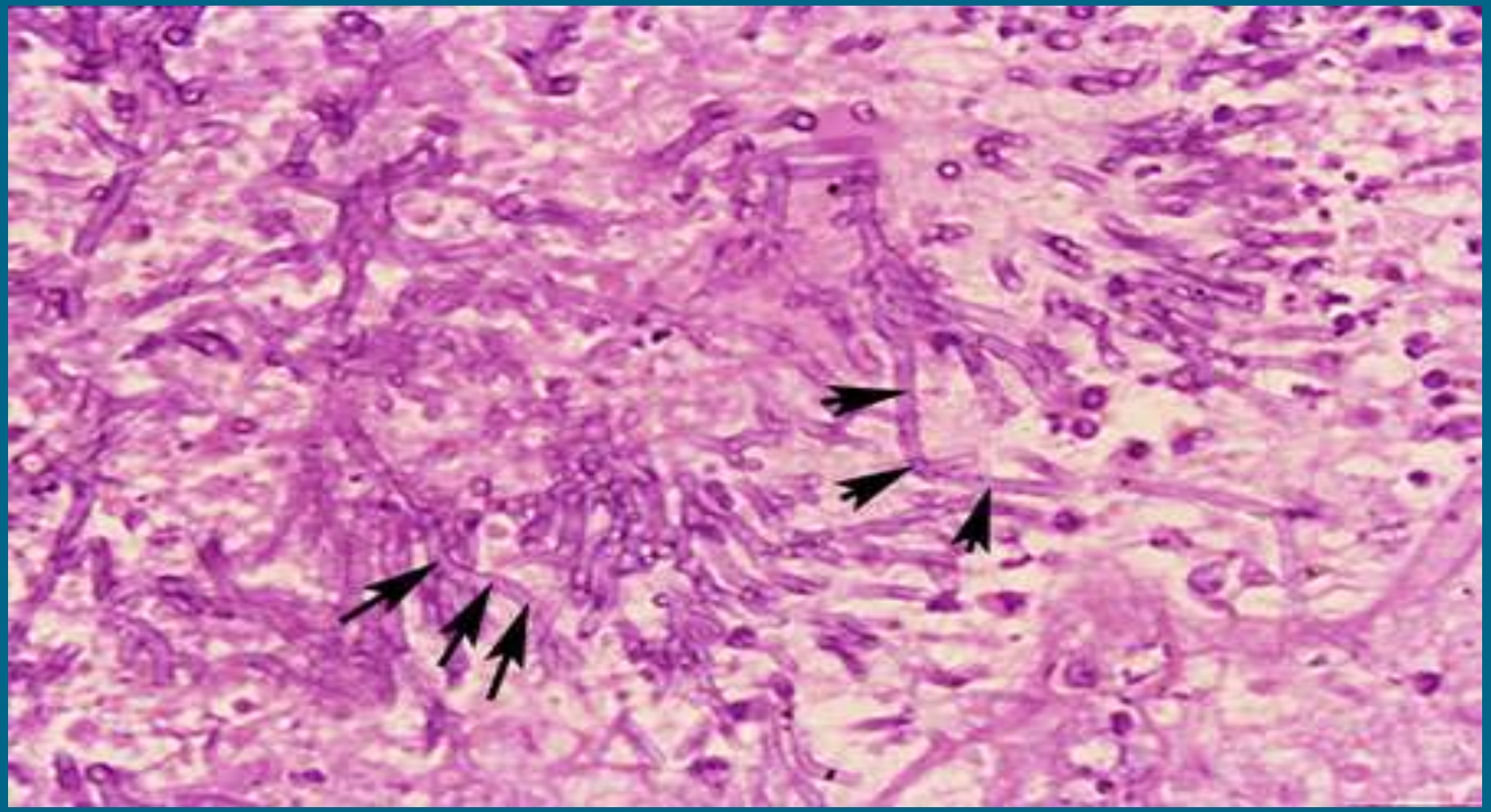
Epidemiology of aspergillosis is tough because……

- EORTC criteria: Probable aspergillosis (50-80% in last clinical trials)
- Proven aspergillosis (50-75%) by microscopic examination only. No ID of species
- Low performance of cultures
- Laboratory contaminants

Molecular diagnostics: biopsy-detection and ID of fungus

Population	Intention	Intervention	SoR	QoE	Reference	Comment
Any	To detect and specify a fungus obtained from a biopsy	Apply molecular analyses in microscopic hyphal-positive and hyphal-negative specimens	A (hyphal positive)	II	Buitrago MJ. CMI 2013, 19:E271-7	In hyphal-positive specimens: High sensitivity (> 90 %) and high specificity (99 %); Various molecular based techniques available.
			C (hyphal negative) Samples	II	Lass-Flörl C. CID 2007; 45: e101-e104	In hyphal-negative specimens: Sensitivity (57 %) and Specificity (96 %) decrease. Ability to distinguish other fungi. Performance only in addition to other tests.
					Lass-Flörl C. Can JM 2011: 57: 765-768	
					Lass-Flörl C. JCM 2012; 51: 863-868	
		In wax embedded specimens	A (hyphal positive)	II	Paterson PJ. MP 2003; 56:368-70	TaKaRa DEXPAT kit and QIAamp DNA mini kit detected less than 10 conidia/sample.
		Species identification of molds in histological tissue sections	A (hyphal positive)	II	Paterson PJ. CID 2006, 42:51-6	Validation and clinical application of molds in tissue. The validity of the method was demonstrated with the establishment of a molecular diagnosis in 52 cases (93%).

PCR in tissues. Proven IFI



ORIGINAL ARTICLE MYCOLOGY

Efficacy of DNA amplification in tissue biopsy samples to improve the detection of invasive fungal disease

M. J. Buitrago[1], J. M. Aguado[2], A. Ballen[2], L. Bernal-Martinez[1], M. Prieto[3], A. Garcia-Reyne[2], J. Garcia-Rodriguez[3], J. L. Rodriguez-Tudela[1] and M. Cuenca-Estrella[1]

1) *Instituto de Salud Carlos III, Majadahonda,* 2) *Hospital Universitario '12 de Octubre', Instituto de Investigación Hospital '12 de Octubre' (i + 12), School of Medicine, Universidad Complutense and* 3) *Hospital Universitario La Paz, Madrid, Spain*

- *84 patients were analyzed*
- *68/84 (81%) were cultured*
- *56% of sensitivity (38/68 cases)*
- *38 cases positives:*

Species	Nb of cases	Rate
Aspergillus	21/38	55%
Mucorales	9/38	23.7%
Candida	7/38	18.5%

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M. J. Buitrago[1], J. M. Aguado[2], A. Ballen[2], L. Bernal-Martinez[1], M. Prieto[3], A. Garcia-Reyne[2], J. Garcia-Rodriguez[3], J. L. Rodriguez-Tudela[1] and M. Cuenca-Estrella[1]

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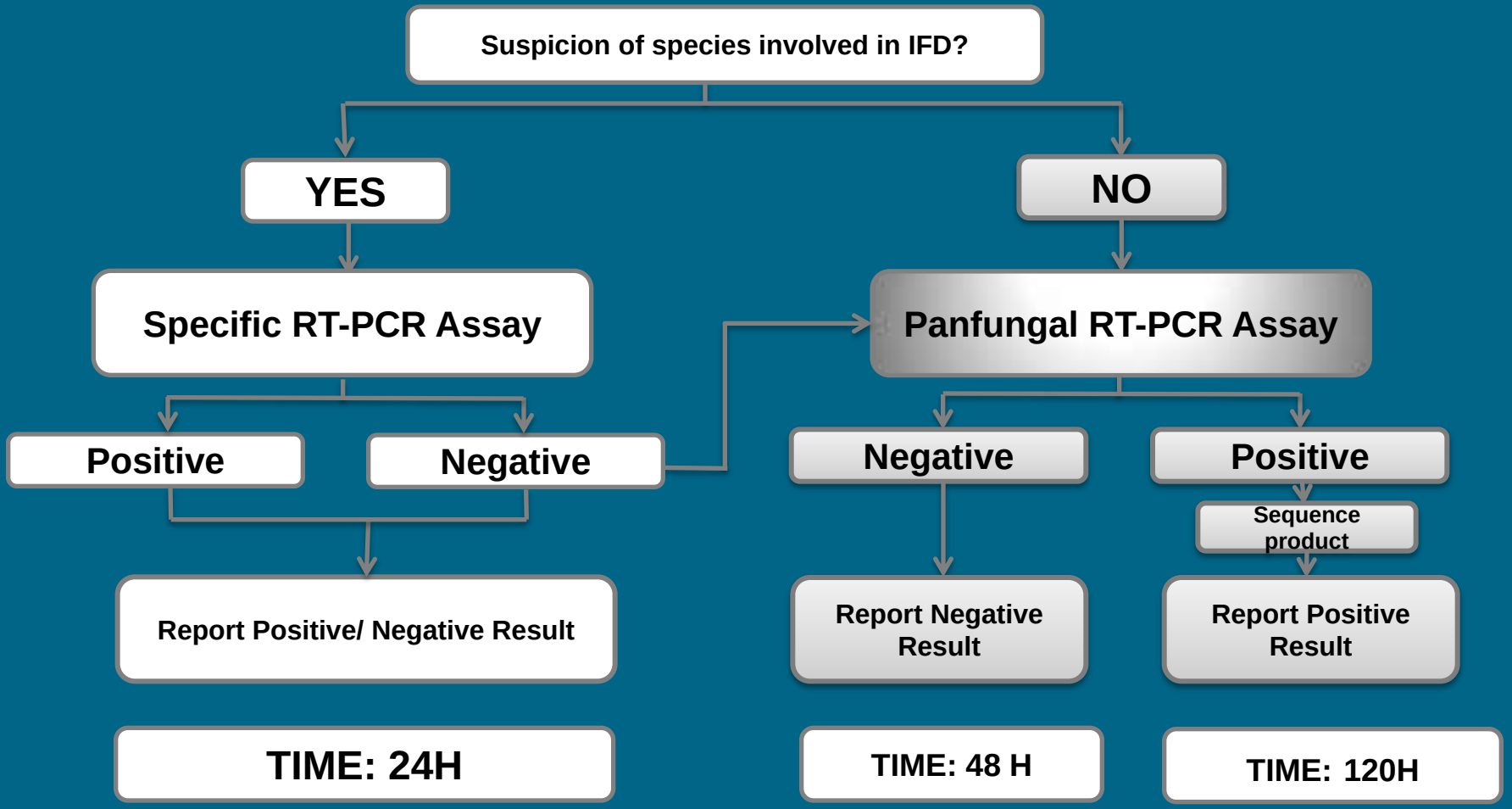
- ***PCR-based technique detected fungal DNA in 75/84 patients (89.3%)***
- ***Six cases, cultures were also negatives***

Species	Nb of cases	Rate
Aspergillus	50/75	66.6%
Mucorales	9/75	12%
Candida	11/75	14.6%

Scheme of procedures performed on biopsy samples
Buitrago et al JCM in press

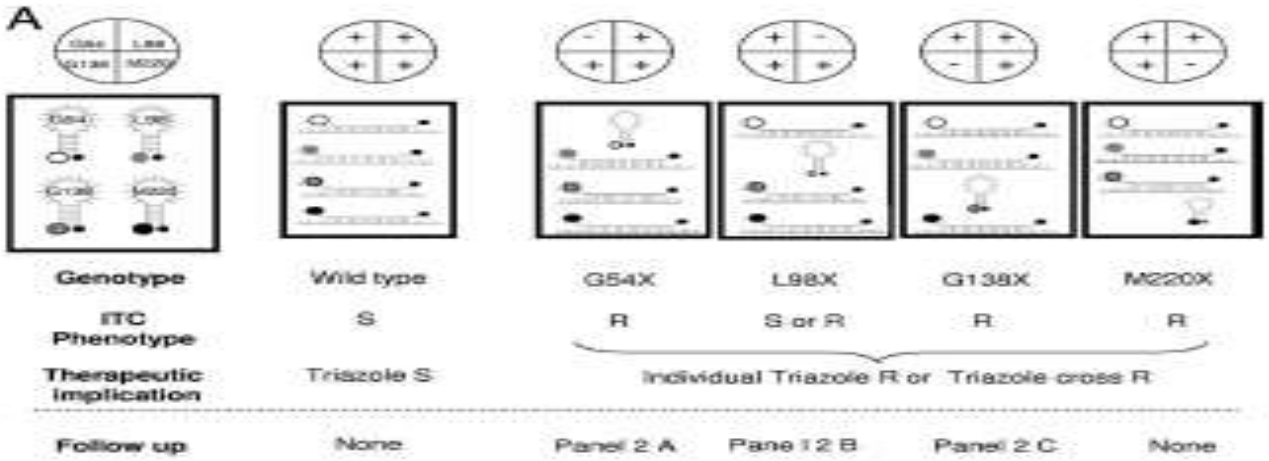
Alternatively, when specific assays were negatives, a panfungal assay was performed



JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2008, p. 1200–1206 Vol. 46, No. 4
0095-1137/08/$08.00+0 doi:10.1128/JCM.02330-07

Rapid Detection of Triazole Antifungal Resistance in *Aspergillus fumigatus*▽

Guillermo Garcia-Effron,[1] Amanda Dilger,[1] Laura Alcazar-Fuoli,[2] Steven Park,[1] Emilia Mellado,[2] and David S. Perlin[1]*



*. Multiplex assay formats: MB panel conformation with all possible results and interpretations. (A) Primary panel. Each quadrant in the

High-frequency Triazole Resistance Found In Nonculturable *Aspergillus fumigatus* from Lungs of Patients with Chronic Fungal Disease

David W. Denning,[1,2,3] Steven Park,[4] Cornelia Lass-Flörl,[5] Marcin G. Fraczek,[2,3] Marie Kirwan,[1,2] Robin Gore,[2] Jaclyn Smith,[2] Ahmed Bueid,[2] Caroline B. Moore,[3] Paul Bowyer,[2] and David S. Perlin[2,4]

Azole resistant *A. fumigatus* Patients' characteristics

- Recurrent infections
- Breakthrough infections
- Tandem repeat mechanism of resistence: 85% mortality
- Evidences from animal models
- Mellado et al, Verweij et al

bjh research paper

Epidemiology and outcome of infections due to *Aspergillus terreus*: 10-year single centre experience

	A. terreus	Other species
Dissemination	63%	32%
CNS	31%	---
Skin	29%	---
AmB response	**20%**	**47%**

Summary for *Aspergillus*

- Increasing rates of azole resistance in *A. fumigatus* (could have cryptic species)
- *A. terreus* R AmB and dissemination
- Molecular identification of *Aspergillus* enlarges its epidemiology
  - More species causing IFI
  - Different profile of AFST

Recommendations for routine daily practice. % of rare species

Sections	%	Remark
Section *Fumigati*	4%	*A. lentulus* is multi-resistant
Section *Terrei*	4%	No enough data
Section *Flavi*	11%	*A. alliaceus,* AMB and echinocandins resistant
Section *Nigri*	50%	*A. tubingensis* , azole resistant
Other rare species	3%	*A. calidoustus*, azole resistant
TOTAL	14% (40/277)	

Recommendations for routine daily practice

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- Classification of species by molecular methods could be useful for clinical management of patients
- 14% of *Aspergillus* clinical isolates are sibling and cryptic species
- Particularly useful in *Fumigatus, Nigri* (*tubingensis* azole resistant) and *Flavi* (*alliaceus* AMB resistant)

Recommendations for routine daily practice

Ann NY Acad Sci 2012

- Surveys to know local epidemiology
- Cases without isolation or non-culturable:
  - Direct detection in clinical samples. PCR-based
  - Inclusion of environmental isolates and relation studies

Recommendations for routine daily practice

Ann NY Acad Sci 2012

- Cases with culture from deep sites:
  - Beta-tubulin sequencing
  - Other possibilities:
    - Matrix-assisted laser desorption/ionization/time-of-flight mass spectrometer. Alanio et al. Clin Microbiol Infect. 2011
    - Luminex assay. Etienne et al. J Clin Microbiol. 2009
  - If not, antifungal susceptibility testing (patient management) and reference center (epidemiology)