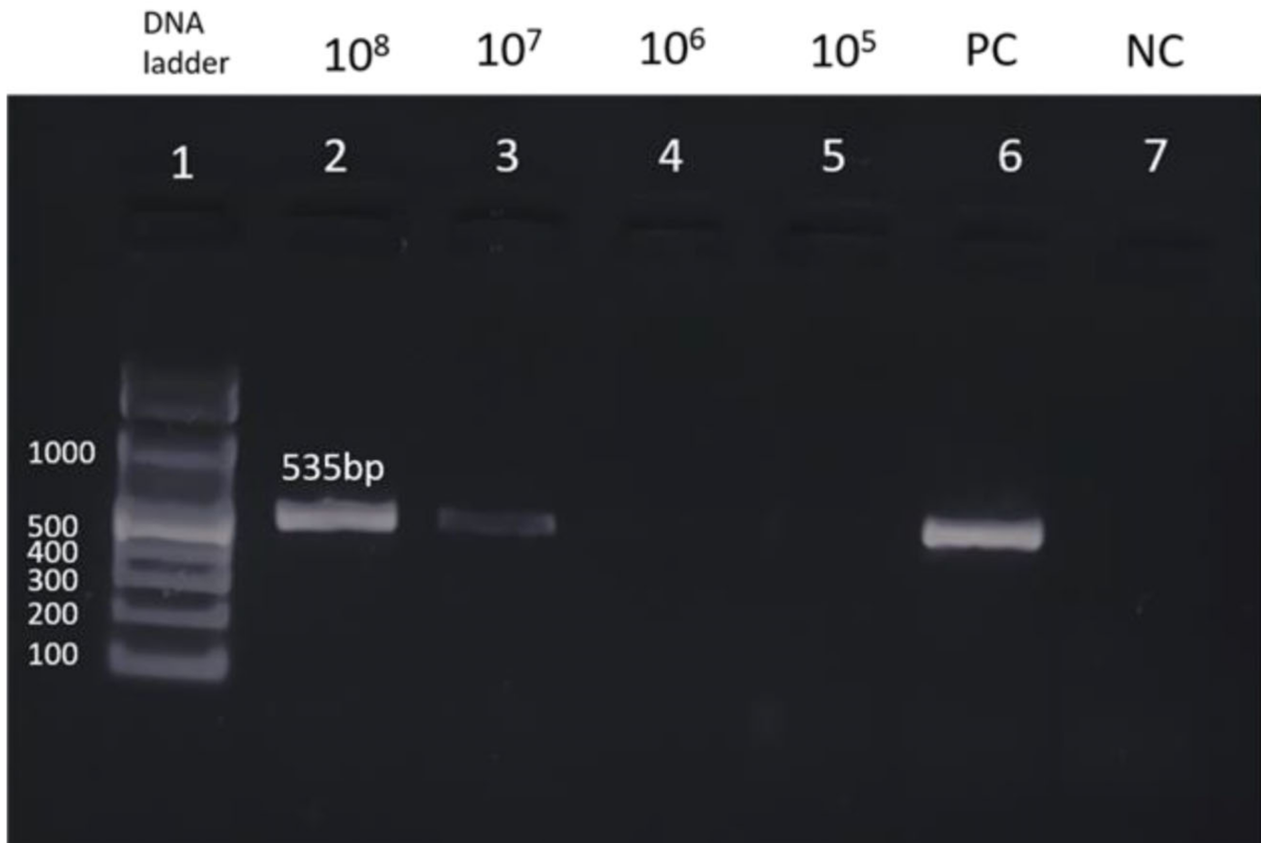

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***Aspergillus* and aspergillosis in patients in an intensive care unit with mechanical ventilation**

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Invasive Pulmonary Aspergillosis (IPA) is a relevant opportunistic disease among neutropenic patients with hematological diseases. Besides them, studies have been showing that critically ill patients in intensive care units (ICU), mainly those infected by respiratory virus such as SARS-CoV-2 are also at risk to be co-infected by *Aspergillus* and developing IPA.

Objectives: We aimed to evaluate the detection of *Aspergillus* in tracheal samples from patients in an ICU on mechanical ventilation from a tertiary hospital from Southern Brazil, and its relationship with their outcome.

Methods: All samples of tracheal aspirate (TA) from patients admitted to the ICU and in mechanical ventilation from July 2020 to June 2021 were included in the study. We performed three different tests to detect *Aspergillus* spp.: (1) mycological culture in Sabouraud Agar Dextrose, with macro and microscopy evaluation of the colonies to the identification of *Aspergillus* section; (2) lateral flow assay for the detection of *Aspergillus* Galactomannan (GM) performed with the cube reader (IMMY® Diagnostics, OK, USA), using a cut-off of ≥4 (nm/ml); (3) quantitative polymerase chain reaction (qPCR) with GoTaq® Probe qPCR (Promega, Wisconsin, EUA) to amplify the small subunit ribosomal RNA target using the forward (3' TTGGTGGAGTGATTTGTCTGCT 5'), and reverse (5' TCTAAGGGCATCACAGACCTG 3') primers, and the probe (3' TCGGCCCTTAAATAGCCCGGTCCGC 5). Samples presenting the cycle threshold (CT) <40 were considered positive. DNA obtained from an *Aspergillus* isolate was used as positive control, and DNA-free water as negative control. Probable aspergillosis was defined in those cases that presented positive results to, at least, two of these tests.

Results: A total of 34 patients were included in the study. Causes of ICU admissions were aids complications ($n = 11$), COVID-19 ($n = 9$), severe acute kidney disease ($n = 6$), tuberculosis ($n = 1$), and other reasons, including post-surgery, septic shock, severe acute respiratory syndrome, and cardiac problems ($n = 6$). *Aspergillus* spp. was isolated in culture of the TA in 50% of the patients (17/34), being 12 *Aspergillus* section *Fumigati*, three *Aspergillus* section *Flavi*, and two *Aspergillus* section *Nigri*. TA from eight patients were positive for GM, and five patients had a positive result in the qPCR assay. Probable aspergillosis was confirmed in 20.6% (7/34), being three patients positive in culture and GM, and three in culture and qPCR. One patient was positive in the three tests. COVID-19-associated aspergillosis (CAPA) corresponded to two of these seven cases. The outcome was death in 13/34 patients, 4 of them (31%) had probable aspergillosis. The other three patients, alive, diagnosed with probable aspergillosis, were treated with amphotericin B, desoxicolate plus itraconazole, and survived. The mortality rate was 57.1% (4/7) and 33.3% (9/27) in the group with and without probable aspergillosis, respectively.

Conclusion: These partial results suggest that aspergillosis can have an important impact in critically ill patients in the intensive care unit of our hospital.

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Massive parallel fungal sequencing on formalin-fixed tissues: development and contribution in integrated histomolecular diagnosis

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Objectives: Histopathology is the gold standard for distinguishing between colonization and fungal infection, but it does not provide a precise diagnosis of genera/species. However, if the culture is negative or if no specimen is sent to the Mycology laboratory, only the specimen sent to the Pathology department is available. Formalin fixation and paraffin embedding (FFPE) cause DNA damage, making it difficult to perform molecular techniques.

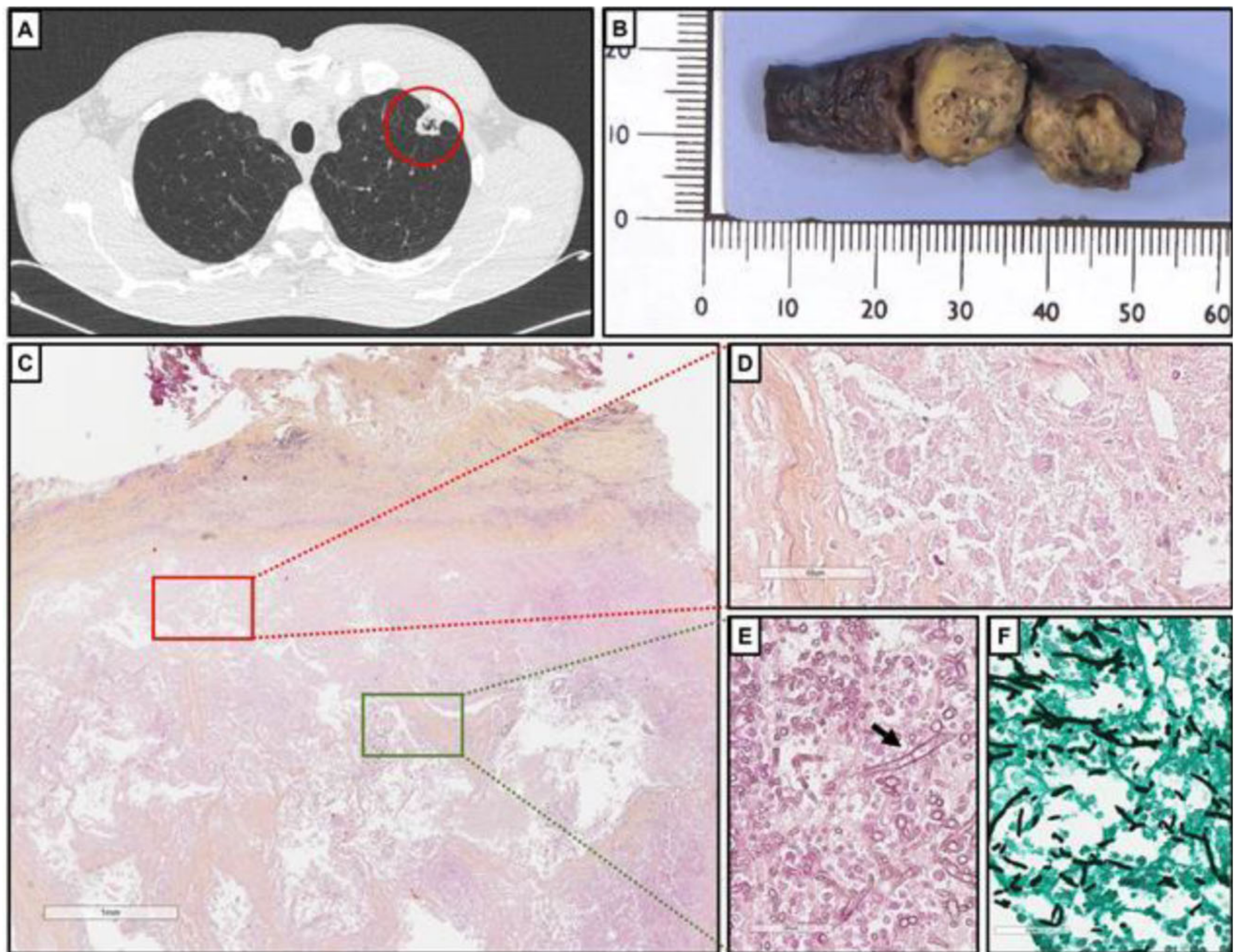
The objective was to develop and evaluate the contribution of massive parallel sequencing (MPS) to FFPE tissues.

Methods: Histopathological review of all cases was performed. Then, DNA extraction from FFPE tissues was optimized by: (1) macrodissecting the fungal-rich area on the paraffin block; (2) comparing the efficiency of two DNA extraction kits (QIAamp DNAmicro-kit, QIAGEN; Maxwell 16 LEVRNA FFPE Purification kit, Promega, optimized for RNA and DNA extraction), by comparison of *Aspergillus fumigatus* and Mucorale specific PCR results for 30 cases. For 124 other cases, the sensitivity of two primer pairs (ITS3/4 and MITS2A/2B) was tested for identification by Sanger sequencing and then MPS. Finally, a histomolecular comparison was performed. This work was funded by the Société de Pathologie Infectieuse de Langue Française (SPILF).

Results: To optimize extraction, DNA was extracted by both kits from samples of 16 mucormycoses and 14 *A. fumigatus* infections. PCR sensitivity was better with the QIAGEN extraction kit [100% (30/30)] compared to the Promega kit [86.7% (26/30)].

PCR amplification of fungal DNA from an additional 124 FFPE samples was performed. The primer pairs ITS3/4 and MITS2A/2B, allowed: (1) identification by Sanger sequencing-histopathological analysis in 38.7% (48/124) of the cases in total, and more specifically 33% (41/124) of cases with the ITS3/4 primers and 32.3% (40/124) of cases with the MITS2A/B primers; and (2) identification by integrated SMP-histopathological analysis in 75% (93/124) of all cases (primers ITS3/4 and MITS2A/2B), and more specifically 66.1% (82/124) for ITS3/4 and 62.1% (77/124) for MITS2A/B (both primer pairs did not detect/amplify the same fungal genera/species). The combination of all results from Sanger sequencing and MPS led to fungal identification in 75.8% (94/124) of cases. In total, the addition of NGS to Sanger sequencing increased the diagnostic proportion by 36.3% (45/124; $P < .0001$). Example of integrated histomolecular diagnosis (Fig. 1): patient with a pseudotumor presentation of pulmonary invasive aspergillosis (A: thoracic CT scan; B: macroscopic examination of lesion after formalin fixation; C, D, E: Hematoxylin-eosin-safran, x20 and x400: observation of a necrotic mass caused by hyalohyphomycetes; F: Grocott, x400) with no culture or molecular identification available on fresh tissue. In contrast, identification by MPS on FFPE tissue was compatible with morphological analysis: *Aspergillus* section Fumigati, leading to the integrated histomolecular diagnosis of pulmonary invasive aspergillosis.

Conclusion: The development of the fungal MPS on FFPE tissues is innovative and unprecedented for the achievement of an integrated histomolecular diagnosis in fungal pathology. It increases significantly the diagnostic proportion by 36.3%. This strategy can be used in hospitals and could improve patient management, especially when no sample is sent to the Mycology laboratory or when the culture is negative.



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Identification of Cryptic species of *Aspergillus* using Beta-tubulin gene in a tertiary care center in South India

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Objectives: To identify cryptic species of the genus *Aspergillus* using Beta-tubulin gene by sequence typing.

Methods: *Aspergillus* grown from various clinical samples (Ear swab, Bronchial wash, Endotracheal Aspiration, Paranasal sinus, BAL, Sputum) was subcultured on Sabouraud's Dextrose Agar/Oatmeal Agar.

Tease mount/Slide culture was done to study the morphological features of the hyphae, size, shape, and arrangement of the conidia.

DNA Extraction using phenol-chloroform method: DNA was extracted and purified directly from 4-day-old culture and used as a template for polymerase chain reaction (PCR) amplification.

DNA Amplification: A fragment of the target gene were amplified using PCR.

The primer pairs used for Beta-tubulin gene was Bt2a (Forward primer) and Bt2b (Reverse primer).

Beta-tubulin.

Bt2a F	GGTAACCAAATCGGTGCTGCTTTC
Bt2b R	ACCCTCAGTGTAGTGACCCTTGGC

DNA Purification: The PCR product was purified with multi-screen filter plates.

Gel electrophoresis: The amplified DNA product was subjected to agarose gel electrophoresis and specific band formation was to be observed for the species of *Aspergillus*.

DNA Sequencing: The purified product will be used as a template for sequencing. An applied biosystem 3730 sequencer will be used to obtain DNA sequences.

Phylogenetic Analysis: Parsimony analysis of individual and combined matrices will be conducted using PAUP version 4.0b10 software/free software at phylogeny.fr by the maximum parsimony (MP) and Neighbor-joining methods.

The results will be represented using a dendrogram.

Results: A total of 30 *Aspergillus* isolates were collected and identified using Phenotypic methods.

The DNA extraction and PCR amplification were done and sent for Sequencing. The results of Sequence typing are awaited.

Conclusion: Cryptic species are morphologically indistinguishable forms of *Aspergillus* and their identifications can be confirmed exclusively by using molecular techniques which have led to the description of previously unknown or rare species among different *Aspergillus* species complex. According to studies, the frequency of such species was found to be 10% in TRANSNET study U.S and 12% in FILPOP study Spain. Most of these species are less susceptible and certain species are multidrug-resistant (azoles and amphotericin B) and the emergence of antifungal-resistance among the *Aspergillus* species forms the importance in identifying Cryptic species.

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Defungi: direct mycological examination of microscopic fungi images

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Objective: To classify five fungi types using two different deep learning approaches and three different convolutional neural network models, VGG16, Inception V3, and ResNet50.

Method: A mycological laboratory in Colombia donated the images used for the development of this research work. They were manually labeled into five classes and curated with subject matter expert assistance. The images were later cropped and modified with automated coding routines to produce the final dataset.

Results: We present experimental results classifying five types of fungi using two different deep learning approaches and three different convolutional neural network models, VGG16, Inception V3, and ResNet50. The first approach benchmarks the classification performance for the models trained from scratch, while the second approach benchmarks the classification performance using pre-trained models based on the ImageNet dataset. Using k-fold cross-validation testing on the 5-class dataset, the best performing model trained from scratch was Inception V3, reporting 73.2% accuracy. Likewise, the best performing model using transfer learning was VGG16, with 85.04% accuracy.

Conclusion: The statistics provided by the two approaches create an initial benchmark to encourage future research work to improve classification performance. Furthermore, the dataset built is published on Kaggle and GitHub to encourage future research.