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ORIGINAL ARTICLE/ARTICLE ORIGINAL

Direct identification of molds by sequence analysis in fungal chronic rhinosinusitis



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Received 13 March 2017; received in revised form 20 June 2017; accepted 17 July 2017

Available online 18 August 2017

KEYWORDS

Rhinosinusitis;
Mold identification;
Sequence analysis;
Culture

Summary Fungi are widely implicated in chronic rhinosinusitis. Direct microscopic examination (DME) is used to confirm the biological diagnosis of fungal rhinosinusitis (FRS). Diagnostic sensitivity of DME is better than culture, however DME does not allow fungal species identification. In this study, we included 54 sinus samples demonstrating hyphae on DME. Direct sequencing was compared to culture for the identification of the fungal species. Sequence analysis identified fungi in 81.5% of cases while culture was positive in only 31.5%. The most common genus was *Aspergillus* and the identified species belonged to section *Fumigati* or to section *Flavi*. Among other fungi identified by sequence analysis, *Schizophyllum commune* was present in three samples attesting to the importance of this Basidiomycetes in FRS. Our results clearly demonstrate the superiority of sequencing compared to culture when performed on specimens with hyphal elements at DME, and contributes to the epidemiological knowledge of fungi involved in FRS.

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Introduction

Chronic rhinosinusitis (CRS) is a very frequent chronic illness affecting all age groups. Current data support CRS as a predominantly multifactorial inflammatory disease with involvement of virus, bacteria or fungi [1–3]. In 5 to 10% of CRS, fungi are responsible for the disease, which encompass a wide spectrum, ranging from noninvasive disease, including fungus balls and allergic fungal disease, to invasive mycosis [4]. Each disease entity has a characteristic presentation and a specific clinical course.

Mycological diagnosis of fungal rhinosinusitis (FRS) is based on the implementation of different methods for the detection of fungi. Table 1 summarizes the different methods for mycological diagnostic procedures, and their respective advantages and drawbacks. The observation of hyphae in sinus samples by direct microscopy examination (DME) confirms the diagnosis [5,6], but does not allow identification of the fungal species involved. The culture of sinus samples can also confirm diagnosis, however, sensitivity is low and depends on the clinical forms of presentation (fungus ball, allergic fungal disease, fungal invasive sinusitis...) [4,7,8]; for example, in FRS with fungus ball, the percentage of positive culture and fungal identification ranges from 10 to 73% [7]. The development of molecular tools had improved detection of fungi in sinus samples, but the success of pathological fungi molecular identification depends on the choice of methods (Table 1). PCR may amplify commensal fungi that have no role in a pathogenic process but are part of the normal microbiota, and non-viable fungal DNA, as well as fungi implicated in infection. Whichever mycological approach is chosen, a final positive result should be interpreted according to the specificity of the method and the radiological and clinical data, in order to distinguish infection from colonization. In addition, it remains important to ensure correct identification of the species involved, especially for invasive FRS, where an appropriate antifungal treatment is promptly required depending on the fungal species.

The aim of this study was to identify fungi involved in FRS based on a positive direct microscopic examination of endoscopically guided sinus aspiration samples (50 samples) and biopsies (four samples). For this purpose, traditional culture methods were compared to direct sequencing of PCR products obtained after amplification of fungal DNA from sinus contents.

Material and methods

Samples

This study was conducted over three years (2011–2013) at the mycology laboratories of Montpellier and Nîmes University Hospitals in southwest of France. During this period, 50 endoscopically guided sinus aspirates samples and four sinus biopsies presenting hyphal elements on DME were included. A total of 54 samples were analysed and frozen at -20°C before DNA extraction and sequencing.

Direct microscopic examination, culture and mycological identification

The presence of hyphal elements was observed by DME after Grocott's methenamine silver stain without centrifugation. Then, samples were plated on Sabouraud chloramphenicol gentamicin agar, with or without actidione, and incubated at 27°C and 37°C for four weeks. Cultures were examined each day during the first week and twice a week thereafter. The identification of fungi from positive cultures was based on macromorphology and micromorphology and confirmed by sequencing for the strains of *Schizophyllum commune*.

Sequencing

DNA extraction

Before DNA extraction, samples were centrifuged with MagNA Lyser Green Beads (Roche) at 6600 rpm during 90 s

Table 1 Mycological methods for fungal rhinosinusitis diagnosis.

Methods	Advantages	Drawbacks
Direct examination [Observation of hyphal elements]	Rapidity, simplicity Specificity (100%): assertion of FRS by presence of hyphal elements	Lack of sensitivity No fungal species identification
Culture [Strain isolation]	Species identification (by morphological examination, or matrix-assisted laser desorption/ionization-time of flight, or DNA sequencing) Detection of fungal mixture Evaluation of antifungal susceptibility	Lack of sensitivity Positive culture: difficult to differentiate colonization to infection Risk of contamination by conidia from the atmosphere during time of culture
Gene amplification [Detection of gender/ species specific fungal DNA, detection of pan-fungal DNA]	Rapidity Fungal species identification: (i) by targeted PCR dependent on the choice of the primers or probes selected; or (ii) by pan-fungal PCR followed by sequencing	Positive PCR: difficult to differentiate colonization to infection Specific PCR: limited number of target species Pan-fungal PCR: no detection of fungal mixture Risk of contamination by amplicon
Next-generation sequencing [Sinus microbiota data]	Identification of a wide range of fungal species Improvement of eco-epidemiology data	Not suitable for routine diagnosis

FRS: fungal rhinosinusitis.

(for disruption). A volume of 200 μ L of supernatant was incubated 2 h with proteinase K at 56 °C. DNA was then automatically extracted (Biorobot[®] EZ1, Qiagen[®]) using EZ1 DNA Tissue kit (Qiagen[®]), according to the manufacturer's instructions and eluted in 100 μ L.

Amplification and sequencing of the fungal internal transcribed spacer 2 (ITS2) region

Fungal ITS2 regions were amplified using the primers MITS2A (5'-GATGAAGAACGCAGCGAAAT-3') and MITS2B (5'-ATGCT-TAAGTTCAGCGGGTA-3'). PCR were performed in a total volume of 50 μ L consisting of 0.2 μ M of each primer, 0.2 mM dNTP, 2.5 U of HotStarTaq[®] DNA Polymerase (Qiagen), 2.5 mM MgCl₂, PCR buffer 10 $\times$ (Qiagen), water and 5 μ L of extracted DNA. PCR were carried out on a Biometra[®] thermocycler using the following program: Taq activation at 94 °C for 15 min, 40 cycles of denaturation (94 °C for 1 min), annealing (58 °C for 30 s) and extension (72 °C for 1 min), and a final extension at 72 °C for 7 min. Amplifications were checked by electrophoresis in 2% agarose gel. The amplicons were then purified using Agencourt[®] AMPure XP (Beckman Coulter). Sequencing reactions were performed using the Big Dye[®] Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions, with MITS2A and MITS2B primers. Sequencing PCR was carried out on a Biometra[®] thermocycler using the following program: denaturation step at 96 °C for 1 min followed by 40 cycles of denaturation (98 °C for 20 s), annealing (50 °C for 10 s) and extension (60 °C for 4 min). The PCR products, after purification with Agencourt[®] CleanSEQ (Beckman Coulter), were run on the Applied Biosystems[®] 3130xl automated sequencer.

Sequence analysis

The ITS2 sequences were aligned using the SeqScape[®] software (LifeTechnologies), adjusted manually by visual inspection and submitted to the CBS database (<http://fr.mycobank.org/>) in order to search homologies with database sequences. For identification at species level, sequence similarities $\geq 99\%$ were required (with 0.8% separation between different species). A sequence was assigned to the genus level if it matched with several species. For *Aspergillus* spp., results were proposed only at section level. Sequences with identification $< 99\%$ were considered to be non-interpretable. For samples with a negative culture, the D1-D2 region of the large-subunit RNA gene was also sequenced.

Primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') were used to amplify this region. The procedures for PCR amplification, PCR product purification, and sequencing of the PCR products were the same as those described for the ITS2 region.

Statistical analysis

A McNemar test was used to compare the differences in percentages with a significance level of $P < 0.05$.

Results

Population characteristics

Among the 54 patients, 38 (70.4%) were female and the median age was 54 years (19–86 years). The samples were distributed regularly throughout each month of the year and no seasonality was found. A final diagnosis of FRS was retained for all the patients, localized to the maxillary sinus in 45 cases (88.3%), sphenoid in four cases (7.4%), ethmoid in four cases (7.4%) and frontal in one case (1.9%).

Direct microscopic examination and fungal culture

Fifty-four samples presented septate hyphal elements on the DME (Fig. 1). Among them, 31.5% (17/54) had a positive fungal culture, with a single fungus. The 17 fungi obtained after culture were identified morphologically to the genus *Aspergillus* in 76.5% (13/17) of cases; seven in the section *Fumigati*, three in the section *Flavi*, one in the section *Nidulantes*, one in the section *Terrei* and one identified as *Aspergillus* spp. The other species identified were *Alternaria alternata* (2/17), *Candida albicans* (1/17) and *S. Commune* (1/17). The *S. commune* positive sample presented typical characteristics of Basidiomycetes on the DME (clamp connections, spicules) (Fig. 1).

Sequence analysis

Among the 54 samples, sequence analysis has identified molds in 44 samples (sensitivity 81.5%). The sequence analysis was non-contributory in 10 samples, two samples did not meet the required 99% sequencing similarities with the database, three products did not have any PCR products

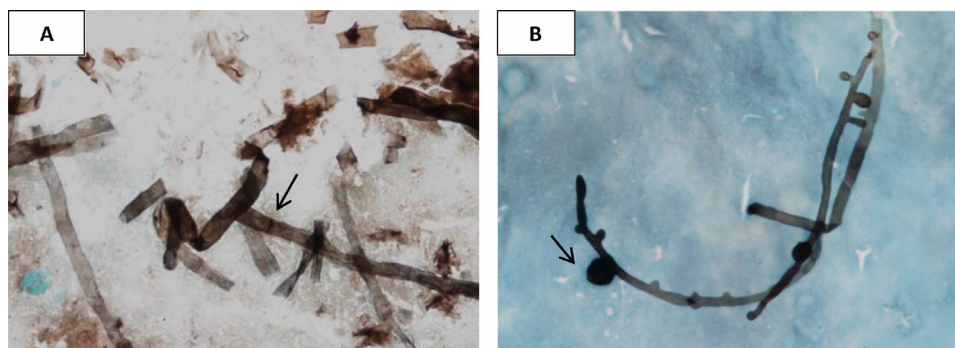


Figure 1 Hyphal elements on direct microscopy examination after Grocott's methenamine silver staining procedure. A. Hyphal elements with septa. B. Clamp connections characteristic of Basidiomycetes).

Table 2 Sequencing results for the 54 sinus samples.

Species identified	Number of samples
<i>Aspergillus</i> spp.	37
Section <i>Fumigati</i>	20
Section <i>Flavi</i>	10
Section <i>Nigri</i>	5
Section <i>Terrei</i>	1
Section <i>Nidulantes</i>	1
<i>Schizophyllum commune</i>	3
<i>Alternaria</i> spp.	2
<i>Fusarium</i> spp.	1
<i>Scopulariopsis brevicaulis</i>	1
Non-contributory sequencing	10
Total	54

and five samples had multiple populations identified in sequence analysis. *Aspergillus* spp. were identified in 84.1% (37/44) cases. The most common species identified belonged to section *Fumigati* or to section *Flavi* for 45.4% and 22.7% of cases respectively. Other species were identified by sequence analysis: *Aspergillus* section *Nigri* (5/44), *S. commune* (3/44), *Alternaria* spp. (2/44), *Aspergillus* section *Terrei* (1/44), *Aspergillus* section *Nidulantes* (1/44), *Fusarium* spp. (1/44), *Scopulariopsis brevicaulis* (1/44) (Table 2).

Concordance between fungal culture and sequence analysis

For 16 sinus specimens, a fungus was identified both by macro and micromorphology of culture and by sequence analysis. Among these sinuses, fungal identification was concordant in 100% of cases (Table 3).

Sequence analysis identified a mold in 75.7% (28/37) of culture negative samples (with positive DME): *Aspergillus* section *Fumigati* (13/28), *Aspergillus* section *Flavi* (6/28), *Aspergillus* section *Nigri* (5/28), *S. commune* (2/28), *Fusarium* spp. (1/28) and *S. brevicaulis* (1/28). For these samples, a second region was sequenced (D1-D2 of the large subunit *rRNA* gene) and confirmed the identification.

For a sample with yeasts and hyphae characteristic of *Aspergillus* spp. on DME, the culture was positive only for *C. albicans* and sequence analysis did not identify any fungi.

A McNemar test demonstrated a statistically significant difference in the proportion of fungi identified by culture and sequence analysis ($P < 0.001$).

Discussion

Molds are widely involved in CRS [9] especially in fungus ball. While DME of sinus samples demonstrates hyphae and confirms the fungal origin of the CRS, more than 50% of cultures remain negative, thus excluding the possibility of fungi identification [7,8].

The aim of our study was to identify fungi involved in FRS by direct sequencing on sinus specimens presenting hyphal elements on DME, compared to culture. Culture was contributive in only 31.5% (17/54) of cases. However, sinus samples were not pretreated with mucolytic agents (dithiothreitol or N-acetyl cysteine) [10,11], which could explain the low rate of positive culture. Direct sequencing has identified fungi in 81.5% (44/54) of cases. Our results were concordant with Willinger et al., 2003 [12], which had previously reported a higher percentage rate of identification by sequence analysis compared to culture (90.3 versus 51.6%).

For 10 samples, sequence analysis was non-contributory. Non-interpretable sequences could be due to limited taxonomy coverage or limited available fungal ITS2 sequences in

Table 3 Comparative results between sequencing and culture of the 54 sinus samples.

Sequencing	Culture	Number of samples
Concordant results	Total	16
<i>Aspergillus</i> section <i>Fumigati</i>	<i>Aspergillus</i> section <i>Fumigati</i>	7
<i>Aspergillus</i> section <i>Flavi</i>	<i>Aspergillus</i> section <i>Flavi</i>	3
<i>Alternaria</i> spp.	<i>Alternaria alternata</i>	2
<i>Aspergillus</i> section <i>Nidulantes</i>	<i>Aspergillus</i> section <i>Nidulantes</i>	1
<i>Aspergillus</i> section <i>Terrei</i>	<i>Aspergillus</i> section <i>Terrei</i>	1
<i>Aspergillus</i> section <i>Flavi</i>	<i>Aspergillus</i> spp.	1
<i>Schizophyllum commune</i>	<i>Schizophyllum commune</i>	1
Discordant results	Total	28
<i>Aspergillus</i> section <i>Fumigati</i>	Negative	13
<i>Aspergillus</i> section <i>Flavi</i>	Negative	6
<i>Aspergillus</i> section <i>Nigri</i>	Negative	5
<i>Schizophyllum commune</i>	Negative	2
<i>Fusarium</i> spp.	Negative	1
<i>Scopulariopsis brevicaulis</i>	Negative	1
Non-contributory ^a	Negative	9
Non-contributory ^a	<i>Candida albicans</i> ^b	1

^a The sequence analysis was non-contributory in 10 samples, two samples did not meet the required 99% sequencing similarities with the database, three products did not have any PCR products and five samples had multiple populations identified in sequence analysis.

^b Sample with yeast and hyphal elements characteristic of *Aspergillus* at the direct microscopic examination (*Candida albicans* could present yeast, hypha or pseudohypha morphotypes).

databases, or perhaps due to fungal mixture. Only the use of methods such as next generation sequencing (NGS) or specific-species PCR allows the detection of fungal mixture.

Regarding species involvement in FRS, *Aspergillus* spp. was the major pathogen (84%, 37/44 cases) found in our study. In the literature, *Aspergillus* spp. is the predominant pathogen in most clinical forms of FRS [8,9]. In fungus ball, *Aspergillus fumigatus* is the most frequently encountered [7,12–14], dematiaceous molds such as *Alternaria* spp. and *Cladosporium* spp. are frequent in allergic forms [8]. A wide range of other fungi can be implicated in invasive FRS, such as Zygomycetes, *Fusarium* spp., *Penicillium* spp. or dematiaceous molds (*Bipolaris* spp., *Curvularia* spp. and *Alternaria* spp.) [8,14]. *Schizophyllum* is the second genus most frequently encountered in our study. Indeed, *S. commune* was identified in three samples (6.8%) by sequencing, but in only one sample by culture. The importance of this basidiomycetous fungus in FRS has been already reported in the literature [12,15–17]. In a recent study, *S. commune* was the second agent responsible for the FRS, encountered in 28.8% of cases [9]. Molecular methods, because of their excellent sensitivity and specificity, provide better information on the diversity of the fungal species involved in FRS. Particularly, NGS enables the study of commensal microorganisms present in the sinus. Indeed, it is now commonly acknowledged that the healthy sinus is not sterile, but is composed of an ecosystem comparable to the lung microbiome containing viruses, bacteria and fungi involved in CRS [11,18]. NGS could lead to a better understanding of the pathophysiology of FRS by comparing the sinusal mycobiota of healthy patients to those of patients with FRS.

Conclusion

Positive DME of endoscopically guided sinus aspiration samples confirms the diagnosis of FRS, but does not allow the identification of fungal species. Culture remains essential to isolate, identify fungal strains and test the antifungal susceptibility, but lacks sensitivity. Our approach using PCR sequencing on positive DME samples identifies a higher percentage of fungi and could provide more robust epidemiological data. Moreover, this approach also achieves rapid results for the installation of targeted treatment in the case of invasive forms.

Disclosure of interest

The authors declare that they have no competing interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mycmed.2017.07.008>.

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Table S1. Results of sequencing analysis of ITS2 region using CBS database (<http://fr.mycobank.org/>)

Tableau S1. Résultats de l'analyse des séquences de la région ITS2 à l'aide de la banque de données CBS (<http://fr.mycobank.org/>)

N° Sample	Reference		Closed reference	
	Identification	Percent identity (sequence length)	Identification	Percent identity
S1	<i>Aspergillus fumigatus</i>	100% (333 nt/333 nt)	<i>Aspergillus fumigatus</i>	99.7%
S2	<i>Aspergillus flavus</i>	100% (334 nt/334 nt)	<i>Aspergillus oryzae</i>	100%
S3	<i>Fusarium proliferatum</i>	99.1% (328 nt/331 nt)	<i>Fusarium fujikuroi</i>	98.2%
S4	<i>Alternaria alternata</i>	100% (318 nt/318nt)	<i>Alternaria pellucida. A. sanguisorbae. A. palandui. A. destruens</i>	100%
S5	<i>Alternaria alternata</i>	100% (326 nt/326nt)	<i>Alternaria tenuissima. A. pellucida. A. palandui</i>	100%
S6	<i>Aspergillus fumigatus</i>	100% (333 nt/333 nt)	<i>Aspergillus fumigatus</i>	99.7%
S7	<i>Aspergillus niger</i>	100% (280 nt/280 nt)	<i>Aspergillus tubingensis</i>	100%
S8	<i>Aspergillus fumigatus</i>	100% (333 nt/333 nt)	<i>Aspergillus fumigatus</i>	99.7%
S9	<i>Aspergillus flavus</i>	100% (277 nt/277 nt)	<i>Aspergillus oryzae</i>	100%
S10	<i>Aspergillus flavus</i>	100% (277 nt/277 nt)	<i>Aspergillus oryzae</i>	100%
S11	<i>Aspergillus terreus</i>	100% (342 nt/342 nt)	<i>Aspergillus terreus</i>	99.7%
S12	<i>Aspergillus fumigatus</i>	100% (332 nt/332 nt)	<i>Aspergillus fumigatus</i>	99.7%
S13	<i>Aspergillus nidulans</i>	99.7% (332 nt/333 nt)	<i>Emericella quadrilineata. E. rugulosa. E. nidulans</i>	99.7%
S14	<i>Aspergillus fumigatus</i>	100% (275 nt/275 nt)	<i>Aspergillus fumigatus</i>	99.6%
S15	<i>Aspergillus fumigatus</i>	99.3% (292 nt/294 nt)	<i>Aspergillus fumigatus</i>	94.0%
S16	<i>Aspergillus fumigatus</i>	100% (333 nt/333 nt)	<i>Aspergillus fumigatus</i>	99.7%
S17	<i>Schizophyllum commune</i>	99% (396 nt/401nt)	<i>Schizophyllum commune</i>	97.5%
S18	<i>Aspergillus fumigatus</i>	100% (333 nt/333 nt)	<i>Aspergillus fumigatus</i>	99.7%
S19	<i>Aspergillus fumigatus</i>	100% (333 nt/333 nt)	<i>Aspergillus fumigatus</i>	99.7%
S20	<i>Aspergillus fumigatus</i>	100% (326 nt/326nt)	<i>Aspergillus fumigatus</i>	99.6%
S21	<i>Aspergillus fumigatus</i>	100% (333 nt/333 nt)	<i>Aspergillus fumigatus</i>	99.7%
S22	<i>Aspergillus flavus</i>	99.6% (276 nt/277 nt)	<i>Aspergillus oryzae</i>	99.3%
S23	<i>Aspergillus fumigatus</i>	100% (276 nt/276 nt)	<i>Aspergillus fumigatus</i>	99.6%
S24	<i>Aspergillus fumigatus</i>	100% (333 nt/333 nt)	<i>Aspergillus fumigatus</i>	99.7%

S25	<i>Aspergillus fumigatus</i>	100% (333 nt/333 nt)	<i>Aspergillus fumigatus</i>	99.7%
S26	<i>Aspergillus flavus</i>	99.6% (274 nt/275 nt)	<i>Aspergillus oryzae</i>	99.6%
S27	<i>Schizophyllum commune</i>	100% (330 nt/330 nt)	<i>Schizophyllum commune</i>	99.7%
S28	<i>Schizophyllum commune</i>	100% (406 nt/406 nt)	<i>Schizophyllum commune</i>	99.7%
S29	<i>Aspergillus fumigatus</i>	100% (333 nt/333 nt)	<i>Aspergillus fumigatus</i>	99.7%
S30	<i>Aspergillus flavus</i>	100% (334 nt/334 nt)	<i>Aspergillus oryzae</i>	100%
S31	<i>Aspergillus niger</i>	100% (334 nt/334 nt)	<i>Aspergillus tubingensis. Aspergillus awamori</i>	100%
S32	<i>Aspergillus niger</i>	100% (334 nt/334 nt)	<i>Aspergillus tubingensis</i>	100%
S33	<i>Aspergillus fumigatus</i>	99.6% (281 nt/282 nt)	<i>Aspergillus fumigatus</i>	99.2%
S34	<i>Aspergillus niger</i>	99.7% (330 nt/331 nt)	<i>Aspergillus tubingensis. Aspergillus awamori</i>	99.7%
S35	<i>Aspergillus flavus</i>	100% (334 nt/334 nt)	<i>Aspergillus oryzae</i>	100%
S36	<i>Aspergillus flavus</i>	100% (334 nt/334 nt)	<i>Aspergillus oryzae</i>	100%
S37	<i>Aspergillus fumigatus</i>	100% (333 nt/333 nt)	<i>Aspergillus fumigatus</i>	99.7%
S38	<i>Aspergillus fumigatus</i>	100% (332 nt/332 nt)	<i>Aspergillus fumigatus</i>	99.7%
S39	<i>Aspergillus flavus</i>	100% (320 nt/320 nt)	<i>Aspergillus oryzae</i>	100%
S40	<i>Aspergillus flavus</i>	100% (334 nt/334 nt)	<i>Aspergillus oryzae</i>	100%
S41	<i>Aspergillus fumigatus</i>	100% (333 nt/333 nt)	<i>Aspergillus fumigatus</i>	99.7%
S42	<i>Aspergillus fumigatus</i>	100% (332 nt/332 nt)	<i>Aspergillus fumigatus</i>	99.7%
S43	<i>Aspergillus tubingensis</i>	99.7% (311 nt/312 nt)	<i>Aspergillus niger</i>	99.7%
S44	<i>Scopulariopsis brevicaulis</i>	100% (349 nt/349 nt)	<i>Scopulariopsis brevicaulis</i>	99.7%