

Fungal Cultures in Patients with Allergic Fungal Rhinosinusitis: Improving the Recovery of Potential Fungal Pathogens in the Canadian Laboratory

Amin R. Javer, MD, FRCSC, Krista A. Genoway, BSc, Mireille Gervais, MD, FRCSC, Marc Romney, MD, FRCPS, and Janet Zemcov, PhD

ABSTRACT

Background: There is no uniform consensus on how to grow fungi from sinus aspirates in the Canadian setting. Protocols vary between institutions, and the positivity rate for fungal cultures ranges between 10 and 20% even when endoscopically obvious allergic mucin is being sent to the laboratory. The aim of this study was to compare the occurrence of positive fungal cultures obtained by our institution's fungal culture method with the occurrence obtained by the Mayo Clinic's fungal culture method. The ultimate aim was to propose a modified, feasible, standardized protocol for culturing fungi from sinus aspirates in the Canadian laboratory setting.

Methods: Twenty-five allergic mucin aspirates were collected in 23 consecutive patients meeting the clinical diagnosis of allergic fungal rhinosinusitis. These samples were sent to the microbiology laboratory, where half of them were subject to our conventional laboratory protocol and the other half to the modified Mayo Clinic protocol.

Results: Positive fungal cultures were obtained in 16 of 25 (64%) specimens when the modified Mayo Clinic culture technique was used, with 12 cultures (48%) growing pathogenic fungus. Using our standard culture technique, 4 of 25 (16%) specimens resulted in a positive fungal culture, of which 3 grew pathogenic fungus (12%). A significantly greater fungal culture yield was obtained by the modified Mayo Clinic fungal culture technique than with our culturing technique.

Conclusion: The modified Mayo Clinic fungal culture technique, although more costly, is a highly sensitive and effective technique for growing fungi from nasal specimens when compared with our traditional culture technique.

SOMMAIRE

Introduction: Il n'y a pas de consensus canadien quant à la meilleure façon de cultiver les fungi obtenus par aspiration sinusale. Les protocoles varient entre les institutions et le taux de positivité des cultures est de 10 à 20% même quand le matériel envoyé apparaît clairement être de la mucine allergique à l'endoscopie. Le but de cette étude était donc de comparer la méthode utilisée de routine dans notre institution à celle proposée par la clinique Mayo. Le but ultime est de proposer un protocole standardisé et réaliste dans les laboratoires canadiens, pour la culture des fungi dans les échantillons sinusiens prélevés par aspiration.

Méthodes: Nous avons aspiré 25 échantillons de mucine allergique chez 23 patients présentant des critères cliniques de rhinosinusite fongique. Ces échantillons ont été envoyés au laboratoire où la moitié a été cultivée en utilisant la technique usuelle et l'autre moitié à une modification du protocole de la clinique Mayo.

Résultats: Nous avons obtenu des cultures positives dans 16 des 25 (64%) échantillons évalués par la modification de la technique de la clinique Mayo dont 12 montraient un fungus pathogène (48%). Notre technique usuelle n'a montré que 4 échantillons positifs (16%) dont 3 étaient des fungus pathogènes (12%).

Conclusion: Notre modification de la technique de culture de la clinique Mayo, bien que plus coûteuse, a démontré une bien meilleure sensibilité pour cultiver les fungi des échantillons nasaux que notre technique traditionnelle.

Key words: allergic fungal rhinosinusitis (AFRS), allergic mucin, fungal culture

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Amin R. Javer, Krista A. Genoway, and Mireille Gervais: St. Paul's Sinus Centre, ENT Clinic, and Marc Romney and Janet Zemcov: Department of Microbiology, St. Paul's Hospital, Vancouver, British Columbia.

Address reprint requests to: Dr. Amin R. Javer, St. Paul's Sinus Centre, ENT Clinic, St. Paul's Hospital, 1081 Burrard Street, Vancouver, BC V6Z 1Y6; e: sinussurgeon@shaw.ca.

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Allergic fungal rhinosinusitis (AFRS) accounts for approximately 7% of all chronic sinus cases requiring surgery.¹⁻³ Prior to its description in the early 1980s by Milar and colleagues and Katzenstein and colleagues,^{3,4} AFRS was often erroneously diagnosed as bacterial sinusitis or nonallergic fungal sinusitis. Even with numerous criteria proposed as of this writing, a universal consensus does not exist for the diagnosis of AFRS. Given that the treatment and prognosis of AFRS differ significantly from other forms of rhinosinusitis, it is extremely important that physicians be able to diagnose AFRS accurately to allow proper management. Recently, Kuhn and Javer suggested useful guidelines for the management of AFRS.⁵

Several diagnostic criteria have been proposed for the identification of AFRS. The major diagnostic criteria proposed by Bent and Kuhn included the presence of nasal polyps; allergic mucin; type I hypersensitivity indicated by history, serologic testing, or a positive skin test; a characteristic computed tomographic (CT) scan; and noninvasive fungus determined by histologic examination or culture.¹ Recently, total serum immunoglobulin E (IgE) levels served as an important indicator of the presence of AFRS, with total IgE values often being elevated in patients with AFRS. In some studies, IgE levels were found to be elevated to more than 1000 U/mL in AFRS patients.⁶⁻⁹ Cody and colleagues proposed a simpler diagnostic criterion for AFRS that required only the presence of allergic mucin and evidence of fungal etiology observed either by the microscopic observation of fungal hyphae in surgical specimens or the recovery of organisms in culture.¹⁰

The ability to grow fungi from specimens in patients suspected of having AFRS is important for obtaining a proper diagnosis.¹¹⁻¹³ Unfortunately, previous studies have shown that less than 50% of specimens collected from patients diagnosed with AFRS have been able to produce a positive fungal culture.¹⁰ Our laboratory has been able to obtain positive fungal cultures only from 10 to 20% of specimens from AFRS patients. Previous studies have indicated that the growth of fungal cultures may, to a great extent, depend on fungal culture methods rather than the presence of fungi in the allergic mucin.⁶ In a 1999 study published by the Mayo Clinic using a novel fungal culture technique, Ponikau and colleagues demonstrated positive fungal cultures in 96% of patients diagnosed with chronic rhinosinusitis.¹⁴ The culture technique proposed by the Mayo Clinic appears to produce significantly greater positive fungal cultures than observed in previous studies. The aim of this study was to compare the rate of positive

fungal cultures obtained by our institution's fungal culture method with the rate obtained by a modified Mayo Clinic protocol using the same culture samples. Ultimately, the aim was to propose a feasible standardized protocol for culturing fungi from sinus aspirates in the Canadian laboratory setting.

Methods

A prospective study of clinically diagnosed AFRS patients at a tertiary sinus centre was performed to compare the occurrence of positive fungal cultures using two culture methods. Patients were excluded from the study if they were immunocompromised, had received antifungal treatment within the previous 6 months, had cystic fibrosis, or were diagnosed with other comorbid diseases (eg, Wegener's granulomatosis, lupus). The clinical diagnosis of AFRS was established if patients had at least three of the five major criteria (excluding positive fungal cultures or histology) as defined by Bent and Kuhn,¹ including (1) nasal polyposis, (2) type I hypersensitivity, (3) allergic mucin, (4) a classic CT scan for AFRS, and (5) a fungal culture or positive histology.

One puff of xylometazoline was sprayed in each patient's nostril to produce vasoconstriction and increase the nasal lumen, allowing for better endoscopic visualization. After 2 minutes, 5 to 10 cc of allergic mucin from the sinus cavity or nose was aspirated into a Leukens trap using a sterile technique and under direct endoscopic visualization. One cubic centimetre of collected specimen was sent for fungal staining and histologic examination, and the remaining specimen was sent to the microbiology laboratory within 30 minutes of collection. All aspirates were handled in a biologic safety cabinet to prevent contamination. The specimen was halved at the laboratory and processed for fungal culture using two differing techniques, as described below.

Modified Mayo Clinic Culture Technique

Half of the collected specimen was processed in accordance with the modified Mayo Clinic culture technique under a laminar flow hood to prevent contamination. The collected specimen was placed in a 50 mL tube with an equal volume of a diluted sterile dithiothreitol, which contained 10 mL of sterile dithiothreitol and 90 mL of sterile water mixture. The mixture was then vortexed for 30 seconds and allowed to stand for 15 minutes to permit the specimen to liquefy. The tube was then centrifuged at 3000g for 10 minutes prior to the supernatant being

removed. The sample was vortexed for 30 seconds before a 0.5 mL prepared sample was inoculated onto an inhibitory mould agar slant containing ciprofloxacin (5 µg/mL); an inhibitory mould agar containing chloramphenicol (125 µg/mL); brain-heart infusion agar containing gentamicin (5 µg/mL), chloramphenicol (15 µg/mL), and 5% sheep blood; and papaic digest of soya bean medium containing chloramphenicol (0.05 g/L) and cycloheximide (0.4 g/L). The slants were grown for 30 days and examined every 2 days. All positive cultures were identified by macroscopic and microscopic means. The slants were all incubated at 30°C in ambient air. The modified Mayo Clinic culturing protocol was identical to that used by Ponikau and colleagues¹⁴ except that our institution did not have access to brain-heart infusion agar containing 5% sheep blood, gentamicin (5 µg/mL), and chloramphenicol (15 µg/mL); and cycloheximide (5 mg/mL). Instead, a comparable plate was prepared of papaic digest of soya bean medium containing chloramphenicol (0.05 g/L) and cycloheximide (0.4 g/L).

Our Standard Culture Technique

Half of the collection specimen was inoculated onto an inhibitory mould agar slant containing chloramphenicol (125 mg/L) and brain-heart infusion agar slants containing 5% sheep blood, chloramphenicol (16 mg/mL), and gentamicin (5 mg/mL). The tubes were incubated at 30°C in an ambient air and read daily for a week for the presence of yeasts and mould. After a week, the tubes were read twice weekly for 4 weeks. All positive cultures were identified by macroscopic and microscopic means.

Statistical analysis was performed for both pathogenic and total fungus grown using mean score analysis and the two-tailed Fisher exact test.

Results

Twenty-three consecutive patients, 15 females and 8 males, with a clinical diagnosis of AFRS were enrolled in this study. Two patients had specimens collected from both the right and left nasal passages, amounting to 25 sinus aspirates. The mean age of the patients was 49 years, with an average of 2.09 surgeries per patient. All patients had polyps, 18 patients had asthma (78%), and 9 patients had acetylsalicylic acid sensitivity (39%). Elevated total serum IgE levels were present in 16 of 22 patients (72%), and eosinophilia was seen in 7 of 14 patients tested (50%).

Of the 25 specimens collected, positive fungal cultures were obtained in 16 (64%) samples using the modified

Table 1. Number of Fungal Organisms Grown from Patients Diagnosed with Allergic Fungal Rhinosinusitis

<i>Fungal Species</i>	<i>Routine Technique</i>	<i>Modified Mayo Clinic Technique</i>
<i>Aspergillus</i>	3	9
<i>Penicillium</i>	0	5
<i>Rhodotorula*</i>	0	3
<i>Candida parapsilosis*</i>	1	2
<i>Stemphylium*</i>	0	1
<i>Cryptococcus*</i>	0	1
<i>Scedosporium*</i>	0	2
<i>Geotrichum*</i>	0	1
Negative	21	9
Total	4	24
Total pathogens	3	14

*Nonpathogens.

Mayo Clinic protocol, of which 12 cultures (48%) grew pathogenic fungus. An average of 0.96 and a maximum of three fungal species were grown per patient using the modified Mayo Clinic protocol. Using our institutional protocol, 4 of 25 (16%) specimens collected resulted in positive fungal cultures, with only one fungal species grown per positive culture. Three of these cultures were identified to be pathogenic (12%), whereas the remaining specimen grew a common saprophyte (Table 1). An average of 0.16 fungal species was grown per patient when our Canadian institution's culturing technique was used. The difference between the two techniques with respect to pathogenic fungi was significant, with a p of .0121 using the two-tailed Fisher exact test.

When the modified Mayo Clinic fungal culture technique was used to grow fungus, the specimens consisted of 37% *Aspergillus* sp, 21% *Penicillium* sp, and 42% saprophytes. Our institution's culture method resulted in positive cultures in 4 of 25 specimens, with 3 being *Aspergillus* sp (75%) and one being a saprophyte (25%).

A significantly greater number of total fungi, both pathogenic and saprophytic, was grown when the modified Mayo Clinic culture protocol was used compared with our institutional fungal culturing technique, as indicated by the Fisher exact test ($p < .001$). When the positive culture rate of pathogenic fungi was analyzed independently, a significantly greater number of pathogenic fungi was grown by the modified Mayo Clinic protocol (12 of 25 vs 3 of 25) compared with our institution's fungal culturing technique, as indicated by the Fisher exact test ($p = .0121$).

Discussion

Acquiring a correct diagnosis is important in determining an effective treatment plan for patients with AFRS. Marple suggested that the inability to grow fungus in patients suspected of having AFRS impedes investigators from forming an appropriate clinical diagnosis.⁶ Although, at present, a positive fungal culture is not felt to be essential for the diagnosis of AFRS, it can be an important contributor to differentiate AFRS patients from patients with other forms of chronic and eosinophilic mucinous rhinosinusitis (EMRS).^{12,13} The treatment strategies may change depending on whether the patient has AFRS or EMRS.^{12,13} Using our protocol, fungus was cultured in only 16% (4 of 25) of the mucin samples collected. Only three (12%) of these specimens grew pathogenic fungi. The modified Mayo Clinic protocol was more successful, with positive fungal cultures being obtained in 64% (16 of 25) of the specimens collected, with 12 cultures (48%) growing pathogenic fungi. These results seem to suggest that the modified Mayo Clinic protocol is superior in its ability to culture fungal specimens when compared with our standard institutional protocol. This may indicate that the inability to produce positive fungal cultures using our method may be largely dependent on laboratory technique and not on the presence of fungus in the patient's mucin. The implication that fungus may be present in the sinuses despite negative culture results draws attention to the need for using a culture method that is sensitive, accurate, and affordable. Our study compared the fungal culture results of two culture protocols, one regarded as a highly sensitive technique¹⁴ and the other regarded as a routine method used in a Canadian tertiary institution. Dividing the fungal aspirates collected in patients meeting the diagnostic criteria for AFRS allows each patient to serve as his or her own control and eliminate many patient-specific variables that can influence fungal culture results.

When the modified Mayo Clinic protocol was used, the specimens grew 37% *Aspergillus* sp, 21% *Penicillium* sp, and 42% saprophytes. These were similar to the results obtained by Lebowitz and colleagues, who grew *Aspergillus* (52%) and *Penicillium* (32%) as the most common organisms in culture-positive patients.¹⁵ The number of positive fungal cultures and distribution of fungi observed in our study were different from those of Ponikau and colleagues, who observed fungal growth in 96% of chronic rhinosinusitis patients,¹⁴ compared with 64% of AFRS patients in our study. Ponikau and colleagues' study had a distribution of 29.5% *Aspergillus* sp, 43.3% *Penicillium* sp, and 55% saprophytes. The difference in the occurrence

and type of fungal species cultured between the two studies could be due to numerous factors, including but not limited to the geographic location of patients, as suggested by Marple,⁶ as well as the significantly different fungal collection methods. Ponikau and colleagues collected specimens in a sterile pan following a patient's forceful exhalation after lavage through the nose.¹⁴ Our study collected all specimens under endoscopic guidance using a Leukens sterile suction trap. The increased positive fungal culture rate, with a greater number of saprophytes obtained by Ponikau and colleagues,¹⁴ may be a reflection of increased contamination from the nasal and vestibule area. Saprophytes accounted for 55% of the total fungus grown in Ponikau and colleagues' study¹⁴ compared with 42% in our study when the modified Mayo Clinic protocol was used. By aspirating mucin directly from the sinuses, our specimens provide a more accurate representation of the fungus within the sinuses, with minimal contamination from the nasal cavity and oral area.

Our modified Mayo Clinic protocol differed from that of Ponikau and colleagues in that we used papaic digest of soya bean medium in preparing one of the plates as opposed to brain-heart infusion agar. In the opinion of our head mycologist, the two media are very comparable and using the papaic digest of soya bean medium should not significantly affect our ability to grow fungus. Nevertheless, the minor change should be mentioned because it is a source of procedural variation.

Our institutional method resulted in positive cultures in 4 of 25 specimens (16%), with 3 specimens being *Aspergillus* sp and one being a saprophyte (Table 1). These results were similar to our centre's overall fungal culture positive rate (12.2%) in patients diagnosed with AFRS as determined from retrospective examination of 140 patients attending our clinic over a 6-month enrollment period.

The laboratory requirements needed to grow fungal specimens using the modified Mayo Clinic protocol are more time consuming and require more safety precautions than our institution's fungal culture protocol. In assessing the additional benefits that the modified Mayo Clinic protocol provides, consideration must be given to the additional requirements of this technique. The additional cost sustained using the modified Mayo Clinic protocol was \$34 per specimen. This can be further broken down into \$9 per specimen for extra supplies and \$25 per specimen for additional labour. The additional labour cost would be expected to decrease significantly if this method were to become routine in the laboratory. Additional requirements for consideration include an increase in

laboratory safety precautions as a result of the use of certain laboratory equipment (centrifugation or vortexing) currently not required in our institution's fungal culture protocol. In our experience, hard and/or dry mucin samples proved to be particularly difficult to culture using the modified Mayo Clinic culture technique. These specimens were difficult to plant in the media and were prone to resist the mucolytic agent, resulting in failure to break the disulphide bonds and liquefy the mucus. It is therefore imperative that all attempts be made to collect moist aspirates when attempting to culture fungi using the modified Mayo Clinic culture technique. It is also useful and recommended that an experienced mycologist be on staff to identify unusual fungi that may be encountered.

These additional expenses and requirements can be substantial and must be considered when assessing the benefits that the more sensitive fungal culture method provides. A handful of reference laboratories in strategic locations across the country may be an ideal way of dealing with the issue of added cost related to the more sensitive modified Mayo Clinic protocol.

Conclusion

The modified Mayo Clinic fungal culture technique is a highly sensitive and effective technique for growing fungi from nasal specimens when compared with our traditional fungal culture technique. Although this technique is more sensitive, it is also more costly and demands additional time and safety requirements.

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