INTRODUCTION

Interactions between fungi and sinonasal tract result in broad spectrum of clinical presentations with varied severity, histologic appearances and biological significance. Fungal Rhinosinusitis (FRS) is categorized histopathologically according to the extent of tissue invasion by fungi into invasive and non-invasive FRS.

Invasive FRS is slightly uncommon, causes tissue destruction, more likely to be associated with ocular symptoms and is potentially life threatening condition that may need early and extensive surgical debridement. Invasive FRS may be acute or chronic invasive and granulomatous invasive FRS.

Non invasive fungal disease includes localized fungal colonization, fungal ball and allergic FRS (ARFS). ARFS is a type of chronic RS which develops secondary to hypersensitivity reactions due to the presence of fungi that become resident in the sinuses. ARFS occurs in immunocompetent patients, this is influenced by climate, geography, and several host factors.

Clinically, AFRS is characterized by being unilateral and expansile process in which mucin contributes to nasal polyp formation, hyposmia/anosmia, and structural changes of the face. AFRS does not invade tissues, but the associated bone loss is related to sinus expansion.

Several randomized trials of topical and systemic antifungal therapies have not shown beneficial results and antifungal therapy for AFRS is unproven. Successful treatment of AFRS virtually always necessitates surgical debridement of the involved sinuses and postoperative steroid therapy to decrease recurrence. The use of postoperative antifungal therapy is still a matter of controversy, where Krishnan et al. supposed the use of antifungal medication as per standard regimen, while Dykewicz et al. documented absence of a firm evidence basis for postoperative use of topical and oral antifungal agents.
Unfortunately, definition and diagnosis of AFRS remain problematic and controversial despite the increasing number of publications. Symptoms are mostly non-specific and colonization is difficult to distinguish AFRS from invasive disease and existing diagnostic tools such as blood cultures, serologic and histopathologic examination are time-consuming methods and often lack sensitivity.

Considering accurate identification of the pathogen is the cornerstone for successful treatment of mycotic infections and filamentous fungi are among the most important pathogens, causing FRs. The current study tried to evaluate the diagnostic outcome of ELISA detection of galactomannan (GM) in sinus lavage aspirate as a rapid minimally invasive diagnostic test for Aspergillus spp. sinus infection in comparison to fungus DNA detection by nested PCR.

METHODOLOGY

Setting:
This cross sectional was conducted at Departments of Otorhinolaryngology and Microbiology at Ibn Sina Colleague Hospitals and Al-Jeddaiini Hospital groups, Jeddah, Saudi Arabia, during the period from June 2016 to August 2018. The institutional review board and the ethical committee has revised and approved this study on March 2016.

Selection of Patients and Sample Collection:
The study intended to include patients presenting to the Outpatient Clinic of Otorhinolaryngology with symptoms suggestive of chronic rhinosinusitis (CRS). CRS was diagnosed according to criteria defined by the Rhinosinusitis Task Force and patients have ≥2 major or one major and ≥2 minor factors for ≥12 weeks despite of medical therapy for 4 weeks were eligible for evaluation. Inclusive criteria: only patients with clinical manifestations suggestive of AFRS were enrolled in the study. Exclusive criteria: patients with a history of malignancies, suspected of having cystic fibrosis, or with previous systemic antismycotic treatment were excluded off the study.

All patients were examined clinically including endoscopic scoring of nasal mucosa edema, presence of secretion and presence of polyps by 0-2 points for a total score range of 0-6 points for each side to determine the extent of the disease. Then, CT scan of paranasal sinuses was performed and resultant scans were scored using Lund-Mackay scale. Each paranasal sinus was graded from 0 (no obstruction) to 2 (complete obstruction) depending on the level of opacification for a total score range of 0-12 points for each side.

All patients underwent bilateral sinonasal irrigation and sampling: after mucosal decongestion, patients were asked to recline their head to 45° and sinus lavage was performed under local topical anesthesia by flushing the maxillary sinus through an 18-gauge spinal needle attached to a collection trap via a 2-way stop to allow flushing with instantaneous sample collection. Needle flushing was performed under the inferior turbinate for each side in all patients.

Laboratory examinations:
Laminar flow was used to process all samples to prevent contamination with airborne fungal spores.

Microbiological examinations:
2–5 ml of the lavage fluids were treated with equal volumes of sterile dithiourethrietol (DTT) (0.3 mg/ml) for 15-min at room temperature to dissolve viscous mucus. Initial screening was done by staining using 10% KOH preparation followed by light microscopic examination. Then, 0.5 ml of the specimens was inoculated on two Sabouraud/dextrose (4%) agar plates (Becton-Dickinson) each containing gentamicin (0.04 g/l) and chloramphenicol (0.4 g/l) were incubated for 4 weeks at 25°C and 37°C respectively, plates were examined daily for fungal growth. Colonies of Aspergillus spp. were identified macroscopically by the color and morphology of growth on agar surfaces and microscopically by staining with lactophenol cotton blue.

Slide cultures were also done, a cover slip was pushed at a 45° angle into a 1-cm square block of potato dextrose agar media (PDA) on the clean sterile slide. When the mould sporulated, the cover slip is carefully withdrawn from the agar and mounted in a drop of lacto-phenol blue on a microscope slide.

Part of the lavage was centrifuged at 3000 rpm for 10 min and about 1.6 ml of supernatant was used for re-suspension of the sediment by vortexing and an aliquot (0.5 ml) was stored at -80°C till be utilized for PCR.

The remaining part of the lavage solution was centrifuged for 20 minutes at 3000 rpm within 30 minutes after sample collection and supernatant was carefully collected and stored at -80°C till be assayed for ELISA detection of galactomannan.

PCR assays:
Fungal DNA extraction:
DNA was extracted from samples by using Quick-DNA Fungal/Bacterial (Cat.No:D6005, Zymo Research, Irvine, CA) Miniprep Kit ZR fungal/bacterial mini prep (Zymo Research) according to manufacturer’s instructions. The extracted DNA concentration was confirmed through measurement by NanoDrop Spectrophotometer2000 (Thermo-Fisher Scientific, Wilmington, USA). Readings were taken at wave lengths of 260 and 280 nm. the purified DNA was stored at -20°C until used in amplification step.

Amplification of DNA by using a nested, two-step PCR technique: according to Skladny et al.

The first round of PCR: was performed using primer AFU7S- (sense primer 5′- CGG CCC TTA AAT AGC CCG -3′) and primer AFU7AS (antisense primer 5′- GA CCG GGT TTG ACC AAC TTT-3′) (Biosearch
technologies, USA). PCR was carried out for 35 cycles consisting of denaturation at 94°C for 45 s, annealing at 65°C for 1 minute and extension at 75°C for 60 s, with initial denaturation 1 cycle of 94°C for 2 minutes and an additional final cycle of extension 5 min at 72°C using Rapid cycler PCR (G Storm Thermal cycler, England). Then the mixture was held at 4°C for the second PCR.

The second round was performed with another sense primer, AFU5S (5′- AGG GCC AGC GAG TAC ATC ACC TTG -3′) and primer, AFU5AS (antisense primer 5′- GG G (AG) GT CGT TGC CAA C (CT)C (CT)CC TGA -3′) (Biosearch technologies, USA). Using 10 μl of the first round PCR. Amplification was obtained by the same method of first round PCR. The PCR reaction mixture contained 25 μl DreamTaq Green in PCR Master Mix (catalogue no: K1081, Thermo Scientific, Germany), 15 μl of tested DNA, 0.5μM of each primer, and nuclease-free water, was added to the PCR mixture to give a final reaction volume of 50 μl. As a negative control in each PCR run, nuclease-free water was used instead of the tested DNA. The PCR products of 1st each round (405 bp), and 2nd round (236 bp) were separated by 2.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV light with molecular . Gene Ruler 100-bp DNA Ladder Plus (Fermentas, Vilnius, Lithuania).

Galactomannan level:
Galactomannan level in undiluted samples was determined using the enzyme linked immunoassay (ELISA) kit (catalogue no. MBS109206, MyBioSource, Inc., San Diego, USA) by sandwich ELISA technique according to the manufacturer’s instructions as follows: All samples were allowed to thaw naturally at room temperature, 50μl of standard, sample and sample diluent were put in standard, sample and Blank/Control well, respectively. Then, 100μl of HRP-conjugate reagent was added to each well; plate was covered and incubated for 60 minutes at 37°C. After 4-time washing, 50μl of each of chromogen solution A and B was added to each well successively, plate was incubated, protected from light, for 15-min at 37°C and then 50μl of stop solution was added to each well. Optical density was read 5-min after adding the stop solution using an ELISA reader at 450 nm.

Statistical analysis:
Possible relationships were investigated using Spearman linear regression analysis and test diagnostic value was calculated. Preoperative investigations were evaluated as predictors for presence of aspergillosis sinus infection in comparison to culture results using the receiver operating characteristic (ROC) curve analysis judged by the area under the curve (AUC) that was compared versus null hypothesis that AUC=0.5. Regression analysis (Stepwise method) was used for stratification of studied parameters as specific predictors. Statistical analysis was conducted using the IBM SPSS (Version 23, 2015) for Windows statistical package. P value <0.05 was considered statistically significant.

RESULTS

During duration of the study, 119 patients presented with picture suggestive of fungal CRS were included in the study; 10 patients refused to have sinus lavage, 4 showed hypertrophy of inferior turbinate that obscured the sinus orifice and 2 patients had complete obstruction of the sinus orifice, so lavage was abandoned; these 16 patients were excluded from the study. Lavage procedure was successfully completed in 95 patients, but the amount of the aspirate fluid was too small to be used for investigation in eight patients and was excluded from the study (Fig. 1). Demographic and clinical data of studied patients (n=95) are shown in table 1.

Fig. 1: Consort Flow sheet
Mean age of studied patients was 39.8±5.5; range: 30-53 years. There were 74 male patients (77.9%) and 21 females (22.1%) with a mean body mass index of 29.8±5.2; range: 24.8-33.9 kg/m² and mean duration of disease of 14.7±6.1; range 7-23 months. All patients had positive endoscopic findings with mean score of 6.4±1.4; range: 5-8 and mean CT score of 15.2±2.4; range: 11-19 (Table 1).

Table 1: Demographic and clinical data of studied patients

<table>
<thead>
<tr>
<th>Data</th>
<th>Findings</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>39.8±5.5</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>74 (77.9%)</td>
</tr>
<tr>
<td>Females</td>
<td>21 (22.1%)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>29.8±5.2</td>
</tr>
<tr>
<td>Duration of disease (months)</td>
<td>14.7±6.1</td>
</tr>
<tr>
<td>Endoscopic score</td>
<td>6.4±1.4</td>
</tr>
<tr>
<td>CT score</td>
<td>15.2±2.4</td>
</tr>
</tbody>
</table>

Data are presented as mean ±SD, numbers & percentages

PCR test defined *Aspergillus* fungal DNA in 56 samples for a detection rate of 58.9%, while culture defined 53 samples as positive for *Aspergillus* spp. infection at a rate of 55.8%. ELISA detection of GM in studied sinus lavage aspirate was positive in 49 samples (51.6%).

In comparison to PCR result, culture showed true positive and negative rates of 43.2% and 28.4% and false result rate of 28.4% and could define cases of fungal sinusitis with sensitivity, specificity, PPV, NPV and accuracy rates of 73.2%, 69.3%, 77.4%, 64.3% and 71.6%, respectively.

ELISA detection of GM, in comparison with PCR results, could diagnose aspergillosis with sensitivity and PPV rates of 84.6% and 89.8% and specificity, NPV and accuracy rates of 88.4%, 82.6% and 86.3%, respectively.

The positive likelihood ratio for culture to diagnose sinus aspergillosis was 2.38 (95% CI= 1.45-3.91), while ELISA detection of GM was 7.28 (95% CI= 3.17-16.72). Moreover, the negative likelihood ratio for culture to exclude sinus aspergillosis was 0.39 (95% CI= 0.24-0.63), while ELISA detection of GM was 0.17 (95% CI= 0.09-0.33), (Table 2).

Table 2: Test validity character of fungal culture and ELISA determination of GM antigen for defining fungal infected aspirate in comparison to PCR test for fungal DNA

<table>
<thead>
<tr>
<th>Test</th>
<th>Data</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPP</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>Value</td>
<td>73.2%</td>
<td>69.3%</td>
<td>77.36%</td>
<td>64.3%</td>
<td>71.6%</td>
</tr>
<tr>
<td>95% CI</td>
<td>59.7-84.17</td>
<td>52.43-82.98</td>
<td>67.52-84.88</td>
<td>52.67-74.44</td>
<td>61.40-80.36</td>
<td></td>
</tr>
<tr>
<td>GM ELISA</td>
<td>Value</td>
<td>84.6%</td>
<td>88.4%</td>
<td>89.8%</td>
<td>82.6%</td>
<td>86.3%</td>
</tr>
<tr>
<td>Detection</td>
<td>95% CI</td>
<td>71.92-93.12</td>
<td>74.92-96.11</td>
<td>79.29-95.29</td>
<td>71.33-90.07</td>
<td>77.74-92.51</td>
</tr>
</tbody>
</table>

ELISA detection of GM significantly correlated with result of culture and PCR (Rho=0.420 & 0.647, respectively, p<0.001) with a positive significant correlation between PCR and culture results (Rho=0.420, p<0.001). ROC curve analysis agreed with correlation analysis but defined ELISA detection of GM as more significant predictor for result of culture with AUC =0.792 (p<0.001) than PCR test (AUC=0.708, p=0.001), (Fig. 2). However, Regression analysis defined PCR testing as the significant predictor for result of culture (β=0.529, p<0.001).

Fig. 2: ROC curve analysis for the predictiveity of ELISA determination of GM antigen and PCR test for fungal DNA for the result of the fungal culture of sinus lavage aspiration fluid
DISCUSSION

This study considered detection of DNA of Aspergillus sp. by nested PCR as a gold standard for comparison of other laboratory diagnostic modalities as primers were designed to have sequences homologous to those of various Aspergillus spp. but not to include the human 18S rRNA gene or the 16S rRNA genes of Candida spp. or other pathogenic microorganisms.

In support of this decision, Malek et al. reported that PCR showed perfect concordance with histology with sensitivity and PPV of 90% and specificity and NPV of 98.3%, but additionally, it diagnosed a sample showed growth of Asp. fumigatus despite the negative histological examination.

Positive PCR for DNA of Aspergillus sp. was obtained in 56 of 95 suspicious samples obtained from CRS patients resistant to standard treatment and prepared for FESS, for a diagnostic ratio of 58.9%; a figure which is coincident with Alanio et al. who detected at least one PCR-positive result in 63.3% of serum samples of invasive aspergillosis (IA) patients, Morio et al. who documented that PCR assay could identify A. fumigatus in 77.4% of patients of fungal rhinosinusitis (FRS) and Rai et al. who detected Asp. flavus infection in 77.5% patients with CRS with nasal polyposis.

These data indicated a role of fungal infection for development of CRS resisting treatment and despite of the curative effect of FESS, the problem necessitated preoperative fungal infection diagnosis is the need for postoperative treatment to minimize the risk of recurrence. However, the traditional reliance on culture is questionable for being time consuming to reach the diagnosis and its low sensitivity rate. Moreover, its specificity was exceeded by the higher specificity of PCR.

In line with this assumption, Miceli & Maertens documented that conventional laboratory diagnostic methods as culture and microscopy, although very useful when positive, are time-consuming and insensitive, resulting in late diagnosis and treatment and contributing to high mortality from invasive aspergillosis (IA).

Moreover, in comparison to positive PCR result, the present study reported true positive and negative rates for culture of 43.2% and 28.4% and false result rate of 28.4%. Similarly, Morio et al. found positive PCR assay in 77.4% of samples compared with 32.1% when using culture as the reference method.

ELISA detection of GM in studied sinus lavage aspirate was positive in 49 samples for a detection rate of 51.6% and in comparison PCR results, ELISA detection of GM could diagnose aspergillosis with sensitivity and PPV of 84.6% and 89.8% and excludes it by NPV of 82.6%.

These data assured results of early studies where Niazi et al. and Swoboda-Kopeć et al. found the molecular methods had high efficiency followed by GM antigen ELISA testing and concluded that real-time PCR assay is the best to identify A. fumigatus; however, the early diagnosis necessitates ELISA and both were useful when IA was suspected. Thereafter, Lehmbacher et al. documented that non-culture methods as detection of GM and molecular tools as PCR may help in the early diagnosis of invasive fungal diseases.

Unfortunately, review of literature showed no similar evaluation of sinus aspirate fluid; however, the obtained results are in line with the results of previous studies evaluated the diagnostic efficacy of ELISA and PCR testing of other body fluids, where Alanio et al. detected a quantitative correlation between serum GM and circulating DNA with an increased possibility of IA when both were positive and Cho et al. found serum GM measurement is valuable in early diagnosis and discrimination of fungal species in patients with AIFR and clinical outcomes may be related to the levels and patterns of serum GM. Also, Winterholler et al. using CSF sample detected A. fumigatus DNA in pan-fungal PCR and sequencing and GM assay was positive, while gave negative microbiological culture.

Concerning accuracy and specificity of ELISA detection of GM, preoperative ELISA detection of GM could define cases of aspergillosis with specificity and accuracy rates of 88.4% and 86.3%, respectively. These test-validity characters allow recommending its application not only as a rapid screening test but also as a diagnostic test; thus could spare the need for culture and histopathology and PCR test may be preserved for assuring diagnosis if required.

These findings go in hand with Arvanitis et al. who documented that negative GM and PCR tests on screening high-risk patients for IA can obviate the need for antifungal agents with NPV of 100%, while the presence of 2 positive results is highly suggestive of an active infection with 88% PPV. Recently, Melancon et al. retrospectively found positive serum GD always correlated with a positive pathologic diagnosis and can indicate AIFR with 100% specificity and PPV, but showed low sensitivity and NPV.

CONCLUSION

Fungal CRS could be considered as a cause for resistance to conventional treatment and so must be investigated prior to surgical-decision taking. Preoperative ELISA determination of GM antigen could identify patients with aspergillosis sinus infection with accuracy and specificity rates of 86.3% and 88.4%. For suspicious cases especially if negative for GM, nested PCR could approach the diagnosis. However, wider scale studies are mandatory to define cutoff point for
GM level in both sinus lavage aspirate and serum level to establish GD determination as one of diagnostic procedures for patients with CRS resisting conventional therapy.

**Conflicts of interest:** The authors declare that they have no financial or non-financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

**REFERENCES**


