Potential EQA for the Detection of Galactomannan in Serum samples: an indicator in the Diagnosis of Invasive Aspergillosis

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Introduction

Aspergillus fumigatus species complex is a filamentous fungus that is the primary causative agent for Invasive Aspergillosis (IA) disease. The production of conidia is a virulence factor that gives this fungus its pathogenicity in humans and almost 200 conidia per day are inhaled by an average person[1]. In healthy individuals, alveolar macrophages phagocytose the conidia and even if some evade the macrophages, infiltrating neutrophils 'mop up' the remainder. This opportunist organism predominantly affects the immunocompromised patients and individuals with underlying risk factors and the disease is characterised by the high mortality rate in these at-risk patients.

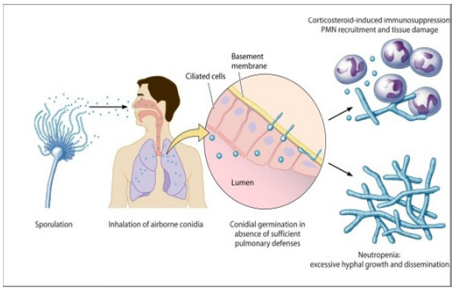

Figure 1*:* Pathway taken by conidia from sporulation to inhalation to germination at the basement membrane of the epithelial cells[1]

Galactomannan (GM) is one of several specific antigens on fungal cell walls which circulate in patient sera during infection. GM is a common aspergilla cell wall polysaccharide, which is released during IA and the link between IA and GM antigen release was first cited in 1979 by Reiss & Lehmann[2].

Several clinical diagnostic laboratories provide a service for determining the concentration of GM in serum samples from patients with suspected IA, a diagnostic test recommended as part of the EORTC/MSG guidelines (2005), in the diagnosis of IA.

Galactomannan detection by the Platelia *Aspergillus* enzyme immunoassay has proven to be a promising tool for the early diagnosis of IA.

Although the manufacturer BioRad does provide a commercial in-house panel of standard control samples for laboratories to test their own performance, there is no externally accredited scheme. There is thus a need for an External Quality assessment programme for laboratories to participate in, who provide a service for the detection of GM.

Several studies have shown the storage of the clinical serum specimens prior to testing may have an impact on the level of GM antigen detected[3].

AIM: To determine the stability of galactomannan in serum, under various storage conditions over a one month period, with view for preparation of simulated serum specimens for distribution in an external quality assessment (EQA) service for clinical diagnostic laboratories.

Materials and Methods

Serum: Serum samples were spiked with an inoculum of a filtered suspension of *Aspergillus fumigatus* species complex.

- Serum (screened negative for HIV, Hepatitis B and Hepatitis C) was tested for GM and only batches that repeatedly produced an index < 0.2 were used to prepare the GM spiked specimens.
- The bulk serum was inoculated with an NCPF strain of *A. fumigatus* species complex and incubated overnight at 37°C aerobically in an orbital shaker (to prevent conidial growth).
- The 'fungal balls' were removed via filtration through a 0.4µm and a 0.2µm filter.
- The serum was inoculated on blood agar and incubated for 24 hours to ensure serum was free of bacterial growth.

Sample preparation:

- Serial dilutions were prepared (from 1: 10^5 to 1: 5 x 10^6) and tested using the Platelia ELISA kit (Biorad), to determine the concentrations of GM in each dilution.
- Dilutions were prepared to obtain final galactomannan indexes (GMI) of >2 (high positive), >1 (positive) , ≤ 0.5 (equivocal) and ≤ 0.2 (negative).
- Serum samples associated with each set of concentrations were dispensed in 1mL volumes into sarstedt tubes and stored at ambient temp (20 - 22°C), 4°C, - 20°C and - 80°C.
- Temperatures were selected according to the storage conditions used by diagnostic laboratories providing a GM detection service.

Sample testing:

- Each set of concentrations stored at the specified storage conditions were tested for GM at 1, 2, 7, 14, 21 and 28 days.
- Each sample was tested in duplicate for the GMI and mean determined to assess the values of GMI over time.

Results

- Serial dilutions up to 1: 5x10^6 were prepared to obtain a final dilution that provided a GMI of ≤ 0.2, as a GMI of < 0.2 was considered as negative.
- Dilution of 1: 5 x 10^6 were interpreted as approximately 1ng/mL: the lowest limit of detection of the antigen with the ELISA .
- Back calculation of the dilutions resulted in the original concentration of the neat filtrate suspension to be approximately 5mg/mL.

Figure 2 illustrates the index determined with 1:100,000 dilution (~ 50ng/mL) maintaining a GMI of >2.0 after 28 days storage in all conditions. Statistics applied showed no significant differences between the different temperatures of storage over the testing period.

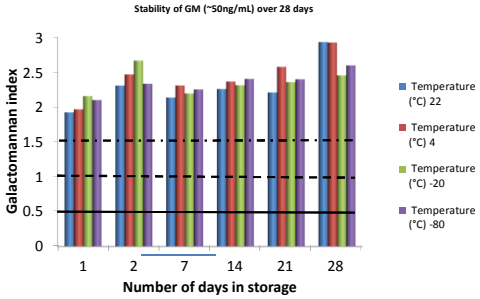

Figure 2*:* Galactomannan index for ~50ng/mL concentration of GM antigen over time under various storage conditions

------ >1.5 index considered cut-off positive for GM (1996)[5]
- - - - >1.0 index considered cut-off positive for GM (2003)[6]
——— >0.5 index considered cut-off positive for GM (2004)[7]

Table 1: GMI of galactomannan concentrations (one way Anova test)

Dilution	temperature °C	Count*	Sum	Average GMI	Variance
1:100000	22	6	13.763	2.294	0.115
~50ng/mL	4	6	14.606	2.434	0.1
	-20	6	14.138	2.356	0.034
	-80	6	14.082	2.347	0.027
1:500000	22	6	11.636	1.939	0.01
~25ng/mL	4	6	11.881	1.98	0.035
	-20	6	12.658	2.108	0.043
	-80	6	12.614	2.102	0.056
1:1000000	22	6	11.349	1.892	0.031
~5ng/mL	4	6	11.807	1.968	0.108
	-20	6	11.402	1.9	0.039
	-80	6	11.394	1.899	0.02
1:2000000	22	6	2.381	0.476	0.001
~2.5ng/mL	4	6	2.668	0.534	0.001
	-20	6	2.715	0.543	0.006
	-80	6	2.747	0.549	0.002

* sets of samples tested

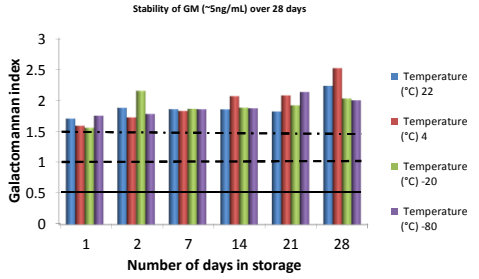

Figure 3*:* Galactomannan index for a ~ 5ng/mL concentration of GM antigen over time under various storage conditions

Figure 3 illustrates the stability of the GM antigen at a concentration of ~5ng/ml and the GMI levels of > 1.5 were determined to consistent throughout the period of storage. It appears a concentration of 5ng/mL have not deteriorated during storage, producing a strong positive GMI at day 28. This value indicates a high level of the antigen in the serum sample.

Figure 4 shows a GMI of ≤ 0.5, a difficult result to interpret. A GMI of 0.5 would require a repeat specimen to confirm the presence of GM in a clinical specimen and proposed treatment of the patient for IA. An excellent stability with reproducibility was demonstrated with levels of GM (~2.5ng/mL).

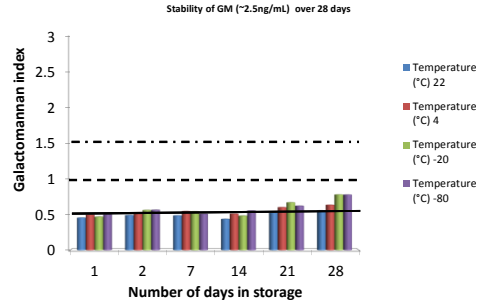

Figure 4*:* Galactomannan index levels for ~ 2.5ng/mL concentration of GM antigen over time under various storage conditions

Discussions

Results of the GMI from the various concentrations of GM determined, showed an excellent stability of cell wall polysaccharide antigen over the set period (28 days) in storage.

An inoculum of 100µl of the culture filtrate into 250mLs of pooled serum, produced approximately 5mg/mL of crude galactomannan. The serial dilutions also showed stability of the GM at low concentrations (2.5ng/mL) and interestingly levels of the antigen increased in value of the GMI when stored at -20°C and -80°C, evaluated at 21 and 28 days. This maybe due to the different lot number of the ELISA kit used to the test kit used for evaluating samples at day 1 – 14, a factor beyond our control for this study.

A one way between-groups analysis of variance was conducted to explore the impact of storage conditions on the levels of GM. There were no significant differences between the different temperatures of storage, as the actual differences in the mean scores between the groups was quite small when tested over the time period (table 1).

Threshold GMI values exhibited low variability between runs which supports consistency in the preparation of the serum samples and the operation of the ELISA test for each set being examined by the operators.

The *Platelia* Aspergillus Ag kit's positive cut off value was originally set at 1.5. There have been several recommendations to reduce the cut-off to 1.0. This reduced GMI would allow patients with a value below 1.5 to be included for treatment for probable or possible cases of IA. The GMI is now recommended to be at 0.5. However from a questionnaire circulated earlier this year (2015) to laboratories who perform this test, revealed that several laboratories still use the 1.5 and 1.0 values as the positive index.

Further work is needed to exam the stability of galactomannan in test samples stored over 6 – 12 month storage, an important factor for advance preparation of specimens for distribution in an EQA programme.

Conclusions

- ❖ Participation in EQA plays a vital role in the quality management and improvement of services offered by clinical laboratories, thereby promoting the ultimate aim of ensuring a high standard of patient care.
- ❖ The range of tests available for mycology services has been expanding and now encompasses; identification and susceptibility testing of yeasts and filamentous fungi, antibody and antibody testing for various bio- markers, DNA detection for a limited species of fungi and a wide range of antifungal assays. There are however still some notable gaps for EQA and one of them being for the detection of galactomannan in serum samples from patients symptomatic for IA.

References

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