Development of an UK NEQAS EQA Scheme: Galactomannan Antigen Detection in Clinical Specimens to Aid Diagnosis of Invasive Aspergillosis Disease

Firat Kartal Shila Seaton Maila Mutso Sanjiv Rughooputh

UK NEQAS for Microbiology, Public Health England, 61 Colindale Avenue, London NW9 5EQ

Introduction and purpose

United Kingdom National External Quality Assessment Service (UK NEQAS) for Microbiology is an external quality assessment (EQA) provider with participating laboratories worldwide. EQA is an invaluable tool for clinical laboratories monitoring the performance, quality and reliability of their service. The purpose of this study was to introduce a new EQA scheme, which would be the first in the industry worldwide, focusing on biomarkers of fungal associated infectious diseases, beginning with galactomannan (GM).

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. Invasive Aspergillosis is initially an infection of the lower respiratory tract but can cause systemic dissemination, without antifungal intervention. Aspergillus fumigatus species complex is the most prevalent fungal pathogen responsible for fatal invasive aspergillosis and immuno compromised individuals (solid organ transplant, hematologic malignancies and neutropenic patients) are most susceptible.

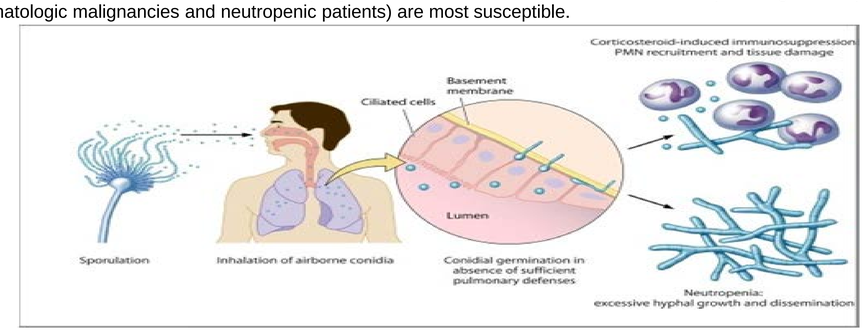

Figure 1: Pathway taken by conidia from sporulation to inhalation to germination at the basement membrane of the epithelial cells [1].

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 and bronchoalveolar lavage (BAL) samples from patients with suspected IA. Detection of the GM, in serum and bronchoalveolar lavage (BAL) is recommended as a criterion in the early diagnosis of Invasive aspergillosis following EORTC/MSG guidelines (2008). Galactomannan detection by the Platelia Aspergillus enzyme immunoassay has proven to be a promising tool for the early diagnosis of IA.

Several studies describe the variability in stability of GM in serum and BAL specimens during storage prior to testing. Some authors describe the GM as very stable at various temperatures however other studies state the antigen deteriorates during storage and recommend testing immediately after receipt of the clinical specimen, to determine the correct level of antigen present in the clinical specimen.

AIMS: To determine the stability of GM in serum and BAL in clinical specimens used for the three pilot distributions.

Methods

BAL and 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) and BAL was tested for GM batches that only repeatedly produced an index <0.2 were used to prepare the GM spiked specimens.
- The bulks were 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 and BAL 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: 10^2 to 1: 5 x 10^4) and tested using the Platelia ELISA kit (Biorad), to determine the concentrations of GM in each dilution.
- Dilutions for specimens 3669, 3670, 3692 and 3716 were prepared to obtain final galactomannan indexes (GMI) of 0.8, 0.1, 1.5 and 1.8 respectively
- Samples associated with each set of concentrations were dispensed in 1mL volumes into 2mL sarstedt tubes and stored at various conditions including +37°C, ambient +22°C, +4°C, - 20°C and - 80°C.
- Each set of concentrations stored at the specified storage conditions were tested periodically in duplicate and the mean GMI values were assessed over time.

Results

From the results obtained specimen 3669 (figure 2) used in pilot 1 was stored for the longest period of time, total 12 months. The highest GMI value obtained over this period of time was 1.127 on day 96 stored at +4°C. The lowest GMI value obtained over the same period of time was 0.551 on day 335 stored at -20°C. The intended GMI on day 0 was 0.851.

Pilot 2 consisted of two serum specimens, the first being specimen 3670 which was not spiked with galactomannan antigen thus the intended result was negative with a GMI of 0.05 (table 1). The second specimen 3692, was spiked with galactomannan antigen thus the intended result was positive with a GMI of 1.5 (figure 3). Both specimens were stored at +4°C for approximately 10 months. The GMI range varied from 0.024-0.235 for specimen 3670 and 1.122-2.186 for specimen 3692.

Pilot 3 consisted of two serum specimens and one BAL specimen, the specimen numbers were 3671, 3715 and 3716 respectively. Specimen 3671, 3715 and 3716 had positive intended results with GMIs of 0.85, 0.66 and 1.76 respectively and were stored for over 3 months at five varying temperatures.

- For specimen 3671 the lowest GMI 0.512 was recorded on day 7 at +37°C and the highest GMI 1.061 was recorded on day 99 at -20°C.
- For specimen 3715 the lowest GMI 0.369 was recorded on day 6 at +37°C and the highest GMI 1.216 on day 71 at +4°C.
- For specimen 3716 the lowest GMI 0.923 on day 6 at +37°C and the highest GMI 2.477 on day 71 at +4°C.

Table 1: A table showing the stability of serum that was not spiked with GM antigen over a period of 10 months (specimen 3670)

Specimen 3670				
Day	Date of testing	GMI Reading 1	GMI Reading 2	Average GMI
0	17.05.2016	0.051	0.051	0.051
41	27.06.2016	0.057	0.052	0.055
72	28.07.2016	0.049	0.057	0.053
101	26.08.2016	0.087	0.044	0.066
129	23.09.2016	0.235	0.053	0.144
155	26.10.2016	0.041	0.036	0.039
189	29.11.2016	0.054	0.054	0.054
211	21.12.2016	0.024	0.035	0.030
241	20.01.2017	0.057	0.07	0.064
276	24.02.2017	0.066	0.071	0.069
303	23.03.2017	0.072	0.127	0.100

Galactomannan Result Interpretation in Serum
≥1.5 index considered cut-off positive for GM (1996) [2]
≥1.0 index considered cut-off positive for GM (2003) [3]
≥0.5 index considered cut-off positive for GM (2004) [4]

Galactomannan Result Interpretation in BAL
≥1.0 index considered cut-off positive for GM [4]

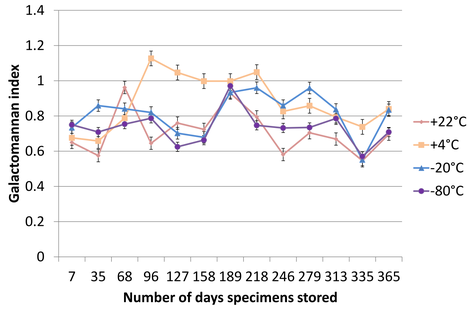

Figure 2: A graph showing the stability of galactomannan antigen in serum over a period of 12 months (specimen 3669)

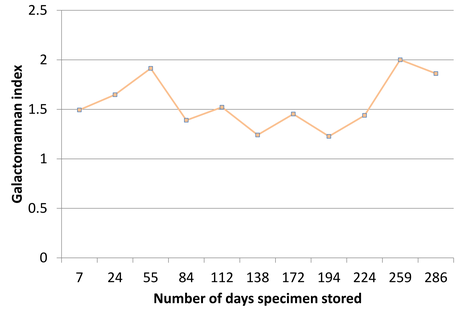

Figure 3: A graph showing the stability of galactomannan antigen in serum over a period of 9 months (specimen 3692)

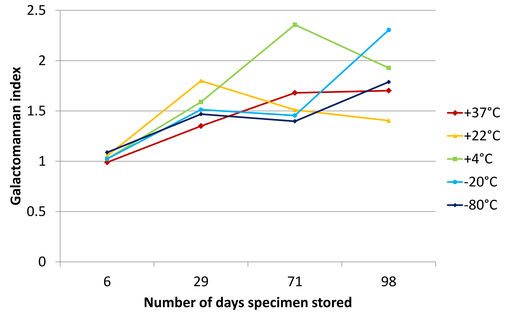

Figure 4: A graph showing the stability of galactomannan antigen in serum over a period of 3 months (specimen 3716)

Discussion

- From the stability results it can be observed that the postive intended results for specimen 3715 and specimen 3716 that were designed close to the cut off 0.5 and 1.0 respectively created a very challenging scenario as some of the results obtained would be interpreted negative according to recommendations.
- At present initial statistical analysis suggests +4°C is the temperature to avoid storing probable positive galactomannan specimens, further analysis needs to be carried out to confirm. All other temperatures used are suitable for storage.
- The Platelia Aspergillus Ag kit's positive cut off value was originally set at 1.5. There were 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 in 2015 to laboratories who perform this test, revealed that several laboratories still use the 1.5 and 1.0 values as the positive index.
- 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

1. Dagenais & Keller, 2009: Clin Microbiol Rev. Jul;22(3):447-65
2. BioRad 1996
3. Pinel et al 2003:J. Clinical Microbiol41(5):2184-6 · June 2003
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