INTRODUCTION

The need for improved diagnosis of invasive fungal disease (IFD) is well documented, and Aspergillus species are the main causes of invasive mould disease. Limited conventional diagnostic methods contribute to high mortality rates, leading to a reliance on empirical therapy. This results in great expense to healthcare providers and unnecessary treatment of, and toxicity for patients (Barnes et al., 2013). The revised EORTC/MSG (European Organisation for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group) criteria have provided standardized definitions for the diagnosis of IFD in clinical trials, and are widely used in clinical practice (De Pauw et al., 2008). Histology is essential to achieve a proven diagnosis but, in clinical practice, particularly ante-mortem, specific radiological signs with supporting mycological evidence (e.g. culture, galactomannan ELISA or β-D-glucan) are used to define probable cases of invasive aspergillosis (IA). PCR is recognized as a potential method for diagnosis but is not included in the criteria because of limited standardization (De Pauw et al., 2008). The literature contains a large number of Aspergillus PCR publications, representing more than 20 years of research (White & Barnes, 2009). The majority of these papers document in-house assays using differing extraction and amplification techniques, highlighting the reasons for excluding PCR from these disease-defining criteria (De Pauw et al., 2008). Over the last 10 years, increasing efforts have been made to standardize Aspergillus PCR through the establishment of several consensus groups and an ever-growing availability of PCR and extraction comparisons (Francesconi et al., 2008; Fredricks et al., 2005; Griffiths et al., 2006; Karakousis et al., 2006; Nawrot et al., 2010).

In 2006, the UK–Irish Fungal PCR Consensus Group established a recommended amplification technique for Aspergillus detection from whole blood (White et al., 2006a). More recently, the European Aspergillus PCR Initiative (EAPCRI) demonstrated that suboptimal extraction efficiency was a major factor in the failure of as many as 50% of participating centres to detect clinically relevant levels of Aspergillus from whole blood (White et al., 2010a). Analysis showed that initial sample input volume, particular sample processing steps (i.e. red and white cell lysis and bead beating) and elution volume all affected PCR performance.
and led to the conclusion that Aspergillus PCR efficiency is limited by extraction and not by PCR amplification (White et al., 2010a, b). This confirmed other research in which conventional extraction methods suitable for extracting nucleic acid (NA) from bacteria and viruses were shown to be insufficient for NA isolation from fungi, and bead beating and increased sample input volume (>600 µl) were suggested as essential (Francesconi et al., 2008; Griffiths et al., 2006; Nawrot et al., 2010). A degree of diagnostic molecular standardization can be achieved by using automated extraction platforms with commercial quality-controlled kits, minimizing inter-operator variability and hands-on time. Fungal NA extraction procedures can be time-consuming and technically demanding, and the use of automated methods is therefore beneficial (Francesconi et al., 2008). Several publications have described the use of automated methods of fungal NA extraction, but direct comparisons of automated systems are limited and all were published prior to the EAPCRI recommendations (Costa et al., 2006; Yera et al., 2009; Dundas et al., 2006; Griffiths et al., 2010a, b; Nawrot et al., 2010). A degree of diagnostic molecular standardization can be achieved by using automated extraction protocols outlined below.

DNA extraction direct from spore suspensions. Quantified conidial suspensions (10³, 10² and 10¹ total conidia) were centrifuged at 10 000 g for 10 min and the supernatant discarded. Conidia were lysed by bead beating using the equivalent of 20–30 µl acid-washed glass beads (Sigma) and vortexing for 30 s. Disrupted conidia were centrifuged at 10 000 g for 30 s to reduce the risk of aerosol dispersion. Disrupted conidia were then subjected to the automated extraction protocols described below.

DNA extraction from EDTA whole blood. Aliquots of EDTA whole blood (3 ml) were spiked with previously determined quantities of A. fumigatus conidia (10³, 10² and 10¹ total conidia per 3 ml EDTA whole blood). Aliquots of blood with no conidia added were used as negative controls. NA was extracted using the EAPCRI method. After bead beating, lysed conidia were transferred to the automated extraction platforms as described below.

Applied Biosystems MagMAX. Extraction of NA was performed following the total NA isolation kit instructions for use (Life Technologies). Disrupted conidia were washed with 120 µl lysis/binding buffer before introduction to the automated NA extractor using the 1840 MagMAX cycle. Samples were eluted in 50 µl elution buffer.

Qiagen EZ1 Advanced XL. EZ1 extractions were processed using the EZ1 DNA tissue kit and card (Qiagen). Following bead beating, 190 µl buffer G2 was used to wash the beads and the washings were added to 10 µl proteinase K for 30 min incubation at 56 °C, prior to addition of samples to the extractor. Samples were eluted in 50 µl elution buffer.

bioMérieux easyMAG. Following bead beating, 200 µl molecular-grade water was used to wash the beads and the washings were transferred to 2 ml NuchiSENS lysis buffer for off-board lysis at room temperature for 10 min. Lysates were then introduced into easyMAG cartridges, after which they were run using the generic 2.0.1 protocol with 60 µl elution volume (bioMérieux).

Roche MagNA Pure LC. The MagNA Pure total NA kit was used in combination with the total NA serum plasma blood protocol (Roche Diagnostics). Subsequent to bead beating, 200 µl molecular-grade water was used to wash the beads and used as sample input. Samples were eluted in 50 µl elution buffer.

PCR amplifications. Aspergillus real-time PCR was performed as described previously (White et al., 2006b). In addition to testing the extraction controls, each PCR assay also included a no-template PCR control to monitor PCR contamination and quantified plasmids containing cloned PCR product to monitor PCR performance and provide a reference threshold for positivity. Internal control PCR was performed as described previously (White et al., 2010a, b).

Patient specimens. Between June 2009 and December 2010, 1202 specimens from 180 patients were prospectively tested. Only data from patients with at least two samples tested are included in this study.

METHODS

Quantification of A. fumigatus. A. fumigatus culture (ATCC 1022) was subcultured on Sabouraud dextrose agar supplemented with chloramphenicol (Oxoid). A dry swab was used to harvest spores from a mature culture after 1–2 weeks of incubation at room temperature. Conidia were subsequently inoculated into 10 ml sterile water with added Tween 20 (Sigma) to reduce conidial clumping. The suspension was subjected to serial dilutions, followed by cell counting (in triplicate) using a Fuchs–Rosenthal haemocytometer to determine conidial concentration in each dilution. While spiking blood with conidia does not accurately reflect the disease process, during which hyphal invasion of tissue and blood vessels occurs, it is not possible to accurately quantify multinucleate hyphae, resulting in inter-specimen variation. A lower limit of 10¹ conidia was deemed to represent a clinically relevant burden, on the basis of crossing point (Cq) comparisons with values typically seen in samples from cases of IA.

DNA extraction. To minimize air-borne contamination, all manual DNA extraction steps were performed in a class 2 laminar flow safety cabinet. All automated extraction protocols were as per manufacturers’ instructions, except where noted. For each instrument, at least three biological replicates were tested for each fungal burden evaluated. All analytical extractions included a negative control to monitor for contamination. A minimum of seven negative controls were tested per instrument. Variability in the number of biological replicates and negative controls used was a result of the testing capacity of the individual instrument combined with the availability of kits and instruments.

All clinical extractions included known positive and negative samples to control for contamination and extraction failure.
study, and the mean number of specimens per patient was 6.7 (median 4, range 2–45). All patients were neutropenic and were undergoing chemotherapy for haematological malignancies. The mean patient age was 37.5 years (range 2–83 years), with a male to female ratio of 1.47. IFD was categorized according to the revised EORTC criteria and included 11 patients with proven/probable IA, 28 patients with possible IA and 123 patients with no evidence of IFD (NEF) (DePauw et al., 2008).

All samples were tested at the request of a consultant haematologist as part of the routine neutropenic fever care pathway and ethical approval was not required. EDTA whole blood and serum were sent for routine fungal PCR and galactomannan ELISA screening (Barnes et al., 2013). NA was extracted from 3–4 ml EDTA whole blood in accordance with the recommended EAPCRI extraction guidelines using the Qiagen EZ1 Advanced XL automated extraction platform for purification (White et al., 2010a). DNA was eluted in 50 μl elution buffer. Positive and negative extraction controls in the form of EDTA whole blood spiked with 101 conidia and a sample with no conidia were included in every diagnostic run to monitor performance. An internal control in the form of a cloned Neisseria meningitidis ctra gene was included to monitor PCR inhibition (White et al., 2010a, b).

Statistical analysis. For each fungal burden tested, the mean real-time PCR Cq values and corresponding 95 % confidence intervals (CIs) were calculated. Mean Cq values for individual burdens were compared using a Student’s t-test, generating two-sided P-values with \( <0.05 \) considered significant. Linear regression analysis was performed to determine the relationship between Cq and the burden of Aspergillus conidia. PCR efficiency was calculated using the following linear equation:

\[
\text{efficiency} = 10^{(-1/\text{slope})}
\]

The limit of detection (LoD) stated refers to the 100 % detection limit.

To evaluate clinical performance, a \( 2 \times 2 \) table was constructed to calculate sensitivity, specificity, positive and negative predictive values, positive and negative likelihood ratios, and the diagnostic odds ratio; 95 % CIs were calculated where necessary. PCR positivity was determined, using a threshold of a single or multiple (two or more) positive results as significant.

RESULTS

A summary of the analytical performance of Aspergillus PCR using DNA extracted by the various platforms is shown in Table 1.

**Applied Biosystems MagMAX extraction**

The LoD when testing quantified conidia was \( 10^1 \) conidia. For EDTA whole blood spiked specimens, the LoD was \( 10^2 \) conidia and the reproducibility of detection for \( 10^1 \) conidia was 25 % (1/4). For all burdens, Cq values were later when testing spiked whole blood compared with the equivalent quantified conidia, but the difference was only significant at the lowest burden (\( 10^1 \) conidia: difference 1.8 cycles, \( P=0.0028 \)). All negative control extracts (10/10) tested were negative for A. fumigatus DNA.

**Qiagen EZ1 Advanced XL extraction**

The LoD when testing conidial suspensions and spiked blood samples was \( 10^1 \) conidia (6/6). Cq values when testing DNA extracted from spiked whole blood were later

### Table 1. Performance of different automated platforms for the extraction of known amounts of A. fumigatus conidia direct from spore suspensions and from spiked 3 ml EDTA whole blood samples

<table>
<thead>
<tr>
<th>Platform</th>
<th>Elution volume (μl)</th>
<th>Detection threshold from culture (total conidia)</th>
<th>Mean Cq at detection threshold (95% CI)</th>
<th>Reproducibility (%) (n/N)</th>
<th>Analytical specificity (%) (n/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MagMAX</td>
<td>50</td>
<td>37.3 (36.7–37.9)</td>
<td>101</td>
<td>100 (4/4)</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td>EZ1 D</td>
<td>50</td>
<td>39.0 (37.2–40.7)</td>
<td>101</td>
<td>100 (6/6)</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td>easyMAG (off-board lysis)</td>
<td>60</td>
<td>36.1 (35.3–37.6)</td>
<td>101</td>
<td>100 (5/5)</td>
<td>90 (9/10)</td>
</tr>
<tr>
<td>MagNA Pure LC</td>
<td>50</td>
<td>38.1 (37.0–39.1)</td>
<td>101</td>
<td>100 (4/4)</td>
<td>100 (7/7)</td>
</tr>
</tbody>
</table>

Cq, Quantification cycle.

*Beads used for in-house lysis/binding buffer supplied with the MagMAX total NA extraction kit before introduction into the MagMAX instrument.

†Employing a 30 min incubation in lysis buffer (GSOL) and proteinase K before introduction into the EZ1 machine.
than those when testing the equivalent DNA extracted from quantified conidia, although the difference was not significant. All negative extracts (8/8) tested during the technical evaluation were negative for A. fumigatus DNA.

**bioMérieux easyMAG extraction**

The LoD when testing conidial suspensions (3/3) and spiked whole blood (4/4) was 10^3 conidia. Differences between the Cq values generated when testing DNA extracted from quantified conidia compared with blood spiked with the equivalent burden were not significant. Of the 10 negative extracts tested, one generated a false-positive result.

**Roche MagNA Pure LC extraction**

The LoD when testing conidial suspensions and spiked blood samples (4/4) was 10^4 conidia. Cq values when testing DNA extracted from spiked whole blood were later than those when testing the equivalent DNA extracted from quantified conidia, although the difference was not significant. All negative extracts (7/7) were negative for A. fumigatus DNA.

**PCR performance**

The standard curves for DNA extracted from 10^3, 10^2 and 10^1 conidia are shown in Fig. 1. PCR efficiencies were 70.83, 100.92, 103.09 and 105.35 % for DNA extracted by the MagMAX, easyMAG, MagNA Pure LC and EZ1 Advanced XL platforms, respectively. Representative PCR efficiency without the influence of DNA extraction and calculated from the mean of a range (300, 30 and 3 copies per reaction) of 10 plasmid replicates was 103.09 %, indicating that the quality of DNA extracted by the easyMAG, MagNA Pure LC and EZ1 Advanced XL platforms had no effect on PCR efficiency, whereas DNA extracted by the MagMAX platform was detrimental to PCR efficiency.

In general, Cq values were earlier when amplifying DNA extracted using the easyMAG platform compared with DNA extracted using the other automated extractors, indicating a better efficiency of extraction (Fig. 1).

**Clinical performance**

Although the easyMAG platform appeared optimal with regard to extraction efficiency, the lower analytical specificity hindered its use in a routine diagnostic setting. Attempts to use the platform for diagnostics resulted in more false-positive results (2/12 negative controls) and were terminated when more than 50 % of samples in a single run, including the negative control, were positive. An internal investigation revealed that buffers 1 and 2 were the source of the contamination (data not shown).

Because the EZ1 platform met the current wider performance requirements for the generic molecular unit and was able to attain the prerequisite detection thresholds for Aspergillus PCR testing of whole blood, it was incorporated into routine service provision. Aspergillus PCR was performed on DNA extracted from 11 patients with proven or probable IA, 28 patients with possible IA and 123 patients with NEF. All proven or probable cases were PCR positive on at least one occasion (100 % of cases, 95 % CI 74.1–100), compared with 75.0 % (95 % CI 56.6–87.3) of possible cases and 28.4 % (95 % CI 21.2–37.0) of NEF patients. Aspergillus PCR performance, with varying degrees of disease definition, is shown in Table 2. The results show that the assay is best used to exclude disease, with consistently high negative predictive values. Conversely, multiple positive PCR results were more frequent in patients with proven/probable IA (72.7 %, 95 % CI 43.4–90.3) than patients with possible IA (39.3 %, 95 % CI 23.6–57.6) and those with NEF (9.8 %, 95 % CI 5.7–16.3), with the difference between proven/probable and NEF cases (62.9 %, 95 % CI 33.0–81.0) reaching statistical significance (P=0.0001).

**DISCUSSION**

Rapid and accurate detection of A. fumigatus DNA has been shown to have potential for improving the diagnosis...
of and outcomes following IA infection in patients with haematological malignancy (White et al., 2006b, 2010a). With the increasing availability of automated extraction platforms, the value of using such platforms to extract A. fumigatus NA is evident, particularly when EDTA whole blood is used, as the process is otherwise laborious and time consuming.

When evaluating an automated extraction robot for use in a clinical microbiology laboratory several factors require consideration, including capacity, target range, efficiency, cost, footprint, level of automation and length of processing time (Table 3). The extraction efficiency of the platform for a given target from the preferred sample type is paramount, and can vary between instruments and even between differing protocols on the same robot (Dundas et al., 2008; McCulloch et al., 2009; Tang et al., 2005; Yera et al., 2009). In this study, the Qiagen, bioMérieux and Roche platforms performed most efficiently in the extraction/purification of A. fumigatus DNA from EDTA whole blood, at clinically significant levels in line with the EAPCRI recommendations (White et al., 2010a). The bioMérieux easyMAG eluates gave detectable levels with consistently low Cq values, suggesting the greatest extraction efficiency (Fig. 1). However, in this study, false positivity through low-level reagent contamination (<20 c.f.u.) excluded the use of the easyMAG platform for isolating A. fumigatus NA from clinical specimens.

In multi-disciplinary molecular laboratories, a range of targets (e.g. virology, bacteriology, mycology and parasitology) and sample types will need to be processed. Robots and protocols should therefore be evaluated against each target and sample type before finally selecting a preferred automated extractor.

Allied to limiting the number of platforms for a variety of targets are the issues of cost, footprint and throughput. The smaller the capital outlay and footprint, the more scope there is for incorporating several robots within a single laboratory. The larger the capital outlay and footprint, the greater the need to select a high-throughput system that is capable of efficiently processing a wide range of sample types and targets.

Of the platforms investigated in this study, the MagMAX has the lowest relative cost and the smallest footprint, but was unable to efficiently extract/purify Cq-derived clinically significant levels of A. fumigatus DNA from EDTA whole blood, despite efforts to optimize procedures. Although compact, the MagMAX extractor allowed up to 24 samples to be processed at one time. However, sample preparation prior to introduction into the robot was a lengthy process and pipetting was problematic due to the low surface tension of reagents, increasing the likelihood of contamination. In contrast, the MagNA Pure LC System has the largest footprint of all the robots and was able to efficiently isolate clinically significant levels of target NA. This platform had the greatest throughput of all the extractors (32 samples) and PCR set-up can also be accomplished by this platform. The EZ1 Advanced XL machine has the second smallest footprint of the robots

Table 2. Performance of Aspergillus PCR when testing clinical samples extracted using the Qiagen EZ1 Advanced XL automated extraction platform

<table>
<thead>
<tr>
<th>Positivity threshold</th>
<th>Value</th>
<th>IA definition*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Single positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity, % (95 % CI)</td>
<td>100 (74.1–100)</td>
<td>82.1 (67.3–91.0)</td>
</tr>
<tr>
<td>Specificity, % (95 % CI)</td>
<td>71.5 (63.0–78.8)</td>
<td>71.5 (63.0–78.8)</td>
</tr>
<tr>
<td>PPV, % (95 % CI)</td>
<td>23.9 (13.9–37.9)</td>
<td>47.8 (36.3–59.5)</td>
</tr>
<tr>
<td>NPV, % (95 % CI)</td>
<td>100 (95.8–100)</td>
<td>92.6 (85.6–96.4)</td>
</tr>
<tr>
<td>LR positive</td>
<td>3.5</td>
<td>2.9</td>
</tr>
<tr>
<td>LR negative</td>
<td>&lt;0.001†</td>
<td>0.25</td>
</tr>
<tr>
<td>DOR</td>
<td>&gt;3500†</td>
<td>11.6</td>
</tr>
<tr>
<td>Multiple positives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity, % (95 % CI)</td>
<td>72.7 (43.4–90.3)</td>
<td>48.7 (33.9–63.8)</td>
</tr>
<tr>
<td>Specificity, % (95 % CI)</td>
<td>90.2 (83.7–94.3)</td>
<td>90.2 (83.7–94.3)</td>
</tr>
<tr>
<td>PPV, % (95 % CI)</td>
<td>40.0 (21.9–61.3)</td>
<td>61.3 (43.8–76.3)</td>
</tr>
<tr>
<td>NPV, % (95 % CI)</td>
<td>97.4 (92.6–99.1)</td>
<td>84.7 (77.6–89.9)</td>
</tr>
<tr>
<td>LR positive</td>
<td>7.4</td>
<td>5.0</td>
</tr>
<tr>
<td>LR negative</td>
<td>0.28</td>
<td>0.57</td>
</tr>
<tr>
<td>DOR</td>
<td>26.4</td>
<td>8.8</td>
</tr>
</tbody>
</table>

DOR, Diagnostic odds ratio; LR, likelihood ratio; NPV, negative predictive value; PPV, positive predictive value.
*Under definition A, proven/probable cases are true positives (n=11), NEF cases are true negatives (n=123) and possible cases (n=28) are excluded from statistical analysis; under definition B, proven/probable/possible cases (n=39) are true positives and NEF cases are true negatives (n=123); under definition C, proven/probable cases (n=11) are true positives and possible/NEF cases (n=151) are true negatives.
†Representative values to replace infinity, calculated assuming sensitivity of 99.9%.
evaluated and was able to perform 14 extractions in one run. The EZ1 required 30 min of off-board lysis prior to purification for optimal extraction efficiency, increasing the total time for extraction. Despite the lower capacity of the EZ1 compared with the other robots, the brevity of the automated portion of the protocol increased the possible daily throughput, demonstrating equivalent performance to that of medium-throughput platforms such as the easyMAG and MagNA Pure LC.

For *Aspergillus* PCR, the sensitivity of the EZ1 platform was comparable with that reported in a previous evaluation (White *et al.*, 2006b), generating a negative predictive value of 97.4–100%, an essential parameter when screening for low-incidence diseases (Barnes *et al.*, 2013). Specificity was lower than in previous studies, but could be improved by using multiple PCR positives as a positivity threshold, and the likelihood ratio positive when evaluating proven/probable IA and NEF cases was 7.4. Approximately two-thirds of PCR-positive NEF patients had non-specific radiological evidence of pneumonia and some may represent cases of IA that are not categorized by the revised EORTC/MSG definitions. Indeed, the original definitions would classify many of these cases as possible IA (Ascioglu *et al.*, 2002).

The emergence of fully automated platforms such as the BD MAX and the Cepheid GeneXpert demonstrate the likely trend for the near future. These platforms automate the whole process of extraction, PCR set-up and PCR, and allow the direct transfer of results to laboratory information-management systems. How these systems perform across a wide range of infective aetiologies, including fungi, remains to be seen. Their potential use in large-throughput laboratories warrants investigation.

In conclusion, we have shown that the Qiagen EZ1 Advanced XL and Roche MagNA Pure LC platforms are suitable for the extraction of *A. fumigatus* DNA from EDTA whole blood using the extraction protocol recommended by the EAPCRI. When incorporated into the screening regimen, the EZ1 Advanced XL platform demonstrated equivalent performance to the previously evaluated MagNA Pure LC platform in terms of negative predictive value (99.3%) on which the service is based. Prior to performing *Aspergillus* PCR subsequent to extraction on automated platforms, full validation of the efficiency of extraction for this target should be undertaken, regardless of validation work carried out for other organisms.

**ACKNOWLEDGEMENTS**

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<table>
<thead>
<tr>
<th>Platform</th>
<th>Capacity (no. samples)</th>
<th>Footprint (cm, W×D×H)*</th>
<th>Off-board lysis (min)</th>
<th>Automated protocol time (min)†</th>
<th>Total protocol time (min)‡</th>
<th>Approximate instrument cost (£)</th>
<th>Cost per sample (£)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambion MagMAX</td>
<td>24</td>
<td>29×39×31</td>
<td>60</td>
<td>15</td>
<td>57</td>
<td>60</td>
<td>9170</td>
</tr>
<tr>
<td>Qiagen EZ1 Advanced XL</td>
<td>14</td>
<td>51×57×57</td>
<td>30</td>
<td>15</td>
<td>45</td>
<td>45</td>
<td>29</td>
</tr>
<tr>
<td>bioMeúrry easyMAG</td>
<td>32</td>
<td>100×65×53</td>
<td>10</td>
<td>45</td>
<td>55</td>
<td>90</td>
<td>38</td>
</tr>
<tr>
<td>Roche MagNA Pure LC</td>
<td>32</td>
<td>108×81×114</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>34</td>
</tr>
</tbody>
</table>

*All dimensions are approximate.
†All times are approximations for capacity processing and do not include operator set-up times, which will increase the total protocol time and will vary according to user.
‡Costs are based on list prices at the time of publication and are for comparative purposes only.
speaker bureaus for Gilead Sciences, MSD, Astellas and Pfizer, and has been sponsored by Gilead Sciences and Pfizer to attend international meetings.

REFERENCES


