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Comparison of Galactomannan Enzyme Immunoassay Performance Levels when Testing Serum and Plasma Samples

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Diagnostic galactomannan (GM) enzyme immunoassay (EIA) testing is formally validated only for serum, though in practice, plasma is occasionally tested. It is assumed, but not confirmed, that results will be comparable to those for serum. GM EIA when testing plasma was evaluated, providing sensitivity (85.7%) and specificity (85.4%) comparable to those for serum. Plasma index values were higher than those for serum; if plasma GM EIA were used to define probable cases, four additional cases would have been diagnosed.

The galactomannan (GM) enzyme immunoassay (EIA) is widely used to aid in the diagnosis of invasive aspergillosis (IA). Serum, plasma, or BAL fluid GM testing is one of the microbiological factors accepted by the revised EORTC/MSG criteria for defining IA (1). GM plasma testing, although recognized by the EORTC/MSG criteria, has not been significantly validated, highlighted by the manufacturer's instructions stating that "the assay has not been evaluated for use with plasma" (2). It is assumed that the two sample types will provide comparable results, but the presence of clotting factors in plasma may increase the adhesive properties of the sample, resulting in potentially higher background optical density values, reducing specificity, and necessitating a higher threshold to define positivity. Alternatively, levels of GM in serum may be reduced by clot formation, potentially making plasma testing more sensitive. No systematic comparison of performance when testing the different samples is available. This study compared the performances of the GM EIA (Bio-Rad) when testing serum and plasma samples in a hematology population.

As part of the local neutropenic fever care pathway, twice-weekly EDTA (4-ml Vacutainer, K2 EDTA spray, catalog no. 367839; Becton, Dickinson) and clotted blood (6-ml Vacutainer, serum tube with no additive, catalog no. 367837; Becton, Dickinson) samples were routinely taken (3). Serum and plasma were prospectively tested by GM EIA and *Aspergillus* PCR, respectively (3). Both samples were stored for internal quality control and performance assessment purposes. Prior to testing, all samples were stored at 4°C. Over a 6-month period, cases (proven, probable, and possible IA) were selected according to disease status as defined, at the time of testing, by the revised EORTC/MSG criteria (Table 1) (1). Controls (no evidence of IA) were taken to coincide temporally with case diagnosis. All paired plasma and serum samples were perfectly matched with regard to sampling time and sample numbers. Plasma samples were retrospectively and anonymously tested by GM EIA according to the manufacturer's instructions with no impact on patient management. Plasma EIA was not included as a microbiology criterion, as testing was retrospective and, although recognized in the EORTC/MSG definitions, the assay has not been validated with this sample type. The study formed an assessment of performance and did not require ethical approval.

Index values for GM EIA when testing plasma and serum were calculated using a positivity threshold of 0.5. When generating mean indices, all values were included. Values for the two samples were compared with regard to overall sample positivity rates (95% confidence intervals [CI] and Fisher's exact test) and mean index values between sample types (paired *t* test). Performance parameters for plasma testing were calculated using 2-by-2 tables. To be considered positive, a patient needed only a single index greater than the threshold. Serum-positive EIA results were confirmed by retesting if the results from plasma and serum were incongruent or if the result represented a single positive among the samples tested per patient and was not confirmed by plasma testing. Otherwise, agreement between samples or multiple positive results were considered confirmation. Three control patients were EIA serum positive on a single occasion, whereas 4 possible-IA patients were EIA plasma positive on a single occasion. Unfortunately, repeat testing of plasma samples was not possible due to limited sample availability.

A total of 284 samples from 65 patients were tested. There were seven cases of proven/probable IA ($n = 1/6$), 10 cases of possible IA, and 48 controls. One proven and two probable cases had *Aspergillus fumigatus* cultured from a respiratory sample. One hundred thirty-five samples were from cases (72 from proven/probable cases [mean, 10.3; standard error of the mean {SEM}, 2.0; range, 3 to 20] and 63 from possible cases [mean, 6.3; SEM, 0.91; range, 1 to 10]), and 149 samples were from controls (mean, 3.1; SEM, 0.42; range, 1 to 12). Overall, there was a trend toward higher sample positivity in cases when testing plasma than when testing serum, but this did not reach significance (proven/probable IA with plasma, 40.3% [95% CI, 29.7 to 51.8]; proven/probable IA with serum, 33.3% [95% CI, 23.5 to 44.8]; possible cases with plasma, 6.3% [95% CI, 2.5 to 15.2]; possible IA with serum, 0% [95% CI, 0 to 5.8]).

False positivity was the same for both sample types (plasma,

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TABLE 1 Clinical performance of galactomannan EIA when testing plasma and serum samples[a]

Parameter	Performance comparison for each pair of sample types with:			
	Plasma testing		Serum testing[b]	
	Proven/probable IA vs no IFD	Possible IA vs no IFD	Proven/probable IA vs no IFD	Possible IA vs no IFD
Sensitivity (%; 95% CI)	6/7 (85.7; 48.7–97.4)	4/10 (40; 16.8–68.7)	6/7 (85.7; 48.7–97.4)	0/10 (0; 0–27.8)
Specificity (%; 95% CI)	41/48 (85.4; 72.8–92.8)	41/48 (85.4; 72.8–92.8)	38/48 (79.2; 65.7–88.3)	38/48 (79.2; 65.7–88.3)
PPV (%; 95% CI)	6/13 (46.2; 23.2–70.9)	10/17 (36.4; 15.2–64.6)	6/16 (37.5; 18.5–61.4)	0/10 (0; 0–27.8)
NPV (%; 95% CI)	41/42 (97.6; 87.7–99.6)	41/47 (87.2; 74.8–94.0)	38/39 (97.4; 86.8–99.6)	38/48 (79.2; 65.7–88.3)
LR+	5.88	2.74	4.11	1.26
LR−	0.17	0.70	0.18	
DOR	35.14	3.91	22.8	

[a] Proven/probable cases ($n = 1/6$), possible cases ($n = 10$), and no-invasive fungal disease (IFD) controls ($n = 48$) were defined using the revised EORTC/MSG criteria with serum GM EIA or *Aspergillus* respiratory culture used as the microbiological criterion. The one case of proven IA was negative by GM EIA in serum and plasma. It was diagnosed as a proven case of IFD, with lung tissue showing septate hyphae with a 45° branching angle, and *A. fumigatus* was cultured from a respiratory sample. Of the 6 probable cases, 4 were serum GM EIA positive only and 2 were serum GM EIA positive and respiratory culture positive. Possible cases had radiological evidence specific to IA but were lacking microbiological evidence. PPV, positive predictive value; NPV, negative predictive value; LR+, likelihood ratio positive; LR−, likelihood ratio negative; DOR, diagnostic odds ratio.
[b] As serum is a disease-defining criterion, an incorporation bias is introduced in favor of serum testing and is evident when analyzing data involving probable cases.

14.1% [95% CI, 9.4 to 20.6]; serum, 13.4% [95% CI, 8.9 to 19.8]). Positivity was significantly greater in proven/probable cases than in controls (serum *P*, 0.001; plasma *P*, 0.0001). False positivity by plasma was in patients (21 samples from 7 patients) that were also falsely positive by serum (17 samples from 7 patients), whereas for serum, additional false positivity was seen in three samples from three patients who were negative by plasma. Five of the seven patients who were falsely positive in both serum and plasma were also positive by *Aspergillus* PCR, compared to one of the three patients who were falsely positive only in serum. Retesting the single EIA-positive serum sample from these three patients generated negative results. Three patients who were falsely positive in both samples comprised 12/20 serum and 17/21 plasma false-positive results. All were PCR positive.

The mean index values for all samples were 0.279 (SEM, 0.04) when testing serum and 0.315 (SEM, 0.045) for plasma. A difference of 0.036 (95% CI, 0.002 to 0.070) and a paired *t* test showed that this difference was significant (two-sided *P*, 0.0398). For proven/probable cases, the mean indices for serum and plasma were 0.758 (SEM, 0.136) and 0.838 (SEM, 0.150), respectively, but the difference of 0.08 (95% CI, −0.008 to 0.166) did not reach significance (two-sided *P*, 0.0742). For possible cases, the mean indices for serum and plasma were 0.109 (SEM, 0.007) and 0.182 (SEM, 0.035), respectively, and the difference of 0.07 was significant (two-sided *P*, 0.0463). For patients without evidence of IA, the mean indices for serum and plasma were 0.158 (SEM, 0.022) and 0.156 (SEM, 0.024), respectively, showing no difference. In terms of qualitative results (i.e., positive/negative), observed agreement between serum and plasma samples was 262/284 (92.3%; 95% CI, 87.1 to 95.5), generating a Kappa statistic of 0.729 (95% CI, 0.582 to 0.877), which represents very good concordance between tests.

The clinical performance of the GM EIA when testing plasma is shown in Table 1. Sensitivity and specificity values are comparable to those for serum for this study and to those previously generated when testing serum (Cochrane meta-analysis; sensitivity, 78% [95% CI, 61 to 89]; specificity, 81% [95% CI, 72 to 88]) (4). The positive and negative predictive values should be interpreted with caution, as they are heavily influenced by prevalence, which can be artificially high in case-control studies. However, in this study, the prevalence of proven/probable disease is 7/65 (10.8%), a value typical of IA in high-risk hematological/stem cell transplant populations. By definition, all possible cases lack microbiological evidence of IA but have radiological features suggestive of fungal disease. Four of the 10 possible-IA cases tested were positive by GM EIA in plasma samples ($n = 4$) and would have resulted in these cases being classified as probable IA if plasma had been prospectively tested.

As serum GM was included within the diagnostic strategy when comparing the performance of plasma with that of serum, this incorporation bias should be adjusted for, but even without adjustment, plasma performance is comparable to that of serum (Table 1). Nevertheless, to overcome this bias, performance was determined using cases diagnosed based on clinical evidence alone, rendering probable and possible cases equal with respect to diagnosis. In doing so, the sensitivity for plasma when testing proven/probable/possible cases is superior to that of serum (58.8% for plasma versus 35.3% for serum; difference, 23.5% [95% CI, 0.3 to 42.5]). Alternatively, the cases may be redefined, retrospectively, using plasma EIA as the microbiological criterion, biasing performance toward plasma and resulting in 11 proven/probable cases with a serum EIA sensitivity of 54.5% compared to 90.9% for plasma (difference, 36.4% [95% CI, −0.59 to 63.7]).

Seven patients with no specific clinical evidence of IA were GM EIA positive in both plasma and serum, and five patients were also positive by PCR. Three patients had multiple positive results in both sample types, all were positive by PCR and had nonspecific radiological evidence, and one had earlier evidence of sinusitis. Using the original EORTC/MSG criteria, designed to determine infection rather than disease, these cases would be considered probable IA (5). This potential to preempt disease will benefit patient prognosis, and these nonspecific signs, supported with specific biomarker evidence, may represent the early infective process (6). None of the three patients with multiple EIA-positive results went on to develop signs specific to IA, although two of the three were treated with antifungal therapy that may have prevented disease.

Conversely, false positivity with GM EIA has been documented, and all patients (cases and controls) would have received piperacillin-tazobactam as part of the neutropenic fever care path-

way (3, 7). As sample positivity rates and mean indices were significantly greater in proven/probable cases than in controls, this is unlikely to have been an issue. Positivity associated with other medical interventions (e.g., PlasmaLyte) was not investigated, although this compound is not extensively used in the local hematology unit.

In conclusion, this study shows that a GM enzyme-linked immunosorbent assay (ELISA) can be performed on plasma specimens using the same positivity threshold and that results generated will be at least comparable to those for serum. Indices for proven/probable/possible cases may be slightly higher than those for serum, resulting in increased positivity and more probable cases being diagnosed. The positivity threshold when testing plasma may potentially need to be adjusted; however, in patients without disease, false-positivity rates and indices are comparable between serum and plasma using the existing threshold. Consequently, clinical performance may be improved compared to that with serum testing, although further investigation is warranted and a prospective cohort study is required to determine accurate clinical performance and designate any positivity threshold adjustments.

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