Donor Derived Cell Free DNA: is it all the same?

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Abstract

Background: The clinical utility of donor derived cell-free DNA (dd-cfDNA) in transplantation has been extensively reviewed, supporting its use as a surveillance tool for the early and accurate detection of allograft injury. Yet studies comparing different assay methods have been lacking.

Methods: Paired sampling of commercially available dd-cfDNA (AlloSure and Prospera) was compared and examined against histology and manufacturer guidance. 76 patients were prospectively assessed, with 11 biopsy proven rejections (ABMR, n=2, TCMR, n=9).

Results: Prospera demonstrated larger measurements of dd-cfDNA in comparison to AlloSure, but this was not significant p=0.12. At current manufacturer recommended diagnostic cutoffs, there was no significant difference in sensitivity, specificity, negative predictive value, or positive predictive value of AlloSure vs. Prospera in detecting rejection. AlloSure demonstrated a significantly shorter turnaround time (p<0.01) from blood draw to patient result.

Conclusion: Although dd-cfDNA assays are similar they are not the same. Extensive evidence for dd-cfDNA interpretation remains key to building clinical utility when considering clinical implementation and remaining consistent to a single platform is important when creating data comparisons.

Introduction

The clinical utility of donor derived cell-free DNA (dd-cfDNA) in transplantation has been demonstrated in the management of patients, where it is has been widely used as a surveillance tool for the early and accurate detection of allograft injury. The validity and utility of this important new biomarker has been extensively reviewed. However studies comparing different assay methods have been lacking, therefore we compared commercially available test services, assessing whether any differences are clinically meaningful.

Two tests commercially available for patient management were evaluated, AlloSure (CareDx, Inc., Brisbane, CA) and Prospera (Natera, Inc., San Carlos, CA). Both have published analytical validation studies but have different degrees of clinical validation. AlloSure has large prospective, multicenter data, while Prospera was substantiated using a single-center, retrospective biobank. Both techniques use proprietary library preparation methods and standard next-generation sequencing (NGS) to quantify dd-cfDNA.

NGS technologies enable sequencing small amounts of DNA in a quantifiable and robust manner, with acceptable time to return diagnostic tests. For NGS assays to discriminate dd-cfDNA from recipient cell-free DNA, polymorphisms between donor and recipient are exploited. Single-nucleotide polymorphisms (SNPs) are the choice for building scalable, high-quality tests to quantify dd-cfDNA, however there is no evidence to show more SNPs assessed is associated with superior outcomes.

Current publications around dd-cfDNA are discordant, with some suggesting superiority and use of a specific library preparation technology. Yet both AlloSure and Prospera use SNP methodology and Illumina-based NGS platforms so it remains unclear whether these claims are true or not in the absence of a head-to-head comparison. The objective of this study is to provide an early real-life multi-center experience comparing results using the commercially available tests to assess these claims using paired values in renal allograft recipients.
Materials & Methods

Sample Collection and dd-cfDNA quantification
Between Nov 2019 and March 2020, 76 single renal transplant patients had simultaneous AlloSure and Prospera dd-cfDNA tests prospectively performed comparing dd-cfDNA levels and reported association with clinical outcomes at either George Washington or within the Dallas Nephrology Associate network. All comers were approached for a paired draw. At the time of study AlloSure was reimbursed by Medicare and being used under a surveillance and for-cause protocol (months 1, 2, 3, 4, 6, 9, 12 within the first year, and every 3 months thereafter), while Prospera was not reimbursable and used only paired with an AlloSure. Dd-cfDNA samples were collected and associated with surveillance visit or renal biopsy post-transplant (Figure 1). Local IRB approval was granted. Interpretation and performance of the tests was based on the manufacturers’ guidance and published validity data for the respective assays. Prospera uses the 1% cut off, where AlloSure uses a gradient, considering relative change above 0.5%. Turnaround time (TAT) from blood draw to the returned test result was recorded. Table 1 compares the two assays.

Patient Demographics and Management
Our typical patient populations are highly sensitized, with PRA>20%, larger AA population, and more frequent HLA-mismatches. The primary causes of end stage renal disease were hypertension and diabetes (Supplement Table 1). The paired design with each patient being their own control and having both tests eliminates concern of confounding factors. All patients had thymoglobulin at 3mg/kg or 1 dose of Simulect at 20mg. Patients were maintained on extended release tacrolimus and mycophenolate mofetil for maintenance, with some weaned from steroids early post-operative, while others had DSA testing done for cause.

Histological reads - Biopsy-proven rejection
Biopsies were read and scored by the local institutional pathologist according to Banff 2017 classification. Results were classified as antibody mediated rejection (ABMR, n=2), T Cell mediated rejection (TCMR, n=9), acute tubular necrosis (ATN, n=4), BK nephropathy (BKVAN, n=1) or normal (n=60). 52 dd-cfDNA tests were ordered as part of surveillance, with 24 ordered due to a change in clinical presentation (formation of de-novo donor specific antibody (DSA), change in creatinine, change in Tacrolimus level, Figure 1).

Statistical Analysis
The Kruskal-Wallis test was used to evaluate the distributions of the difference between the paired dd-cfDNA percentages (AlloSure – Prospera) across the categories of biopsy occurrence. The Wilcoxon signed rank test was used to evaluate the difference between the paired turnaround times between the assays. The method outlined by Hanley and McNeil was used to derive sample size estimates based upon comparing AUC ROC for paired data. All statistical tests reported are two-sided, where statistical significance is defined as a p-value less than 0.05.

Results
A total of 76 samples were assessed with both AlloSure and Prospera, which included 11 (14%) biopsy proven rejections. Measurements of dd-cfDNA between the testing methods for the paired samples (excluding 1 paired sample with AlloSure score of > 16 and Prospera score of 19.61) tend to demonstrate larger measurements for Prospera in comparison to AlloSure as shown in Figure 2A (0%-1%) and Figure 2B (0%-20%), (Kruskal-Wallis test chi-square=2.59, df=2, p=0.27). This trend was most apparent for samples with associated biopsy confirmed rejections (Figure 2C).
dd-cfDNA as a molecular marker of injury may be elevated by other causes, these include BKVAN. The one patient with BKVAN had SV40 staining on biopsy with blood-based PCR load of 100,000 copies. AlloSure has evidence suggesting the degree of injury can differentiate viremia from nephropathy, but the sample is small with active trials underway. No significant differences amongst diagnostic test characteristics were observed, despite AlloSure trending higher performance. This is based upon the estimates obtained and their associated 95% confidence intervals (Table 2, Table 3A-3B and Table 4). A prohibitively large number of samples would be required to delineate the nominal differences, which are not clinically relevant. For example, the estimated AUC ROC for AlloSure and Prospera are 0.73 and 0.75, respectively. To demonstrate AUC ROC superiority of Prospera relative to AlloSure, assuming a significance level of 5%, power to detect a difference of 80%, correlation among the paired samples of 96% and 15% of the biopsies performed resulted in rejection, the estimated number of samples that would need to be collected, tested across both diagnostic platforms, and have biopsies performed to confirm rejection is more than 2300. Differences between the assays matter most for accurately detecting all types of rejection. Prospera missed 3 cases of TCMR1A using the 1% threshold which were detected by AlloSure using its published guidance of 0.5%. Even at 0.5%, Prospera missed two TCMR1A cases, which were identified by AlloSure.

The most meaningful difference captured in this study is the amount of time from sample collection to the availability of the reported results. Figure 3 displays the distribution and cumulative distribution of the difference in turnaround time between the paired samples. 75% of the paired samples had a difference of at most -1 day. This indicates that 75% of the AlloSure tests were reported at least 1 day earlier than Prospera tests (P<0.01, Wilcoxon signed rank test, Figure 3), with the largest difference being 5 days for 2 samples. There were 3 samples where the AlloSure samples took longer to report than the paired Prospera samples, with a difference of a single day.

**Discussion**

While AlloSure and Prospera both use the novel clinical parameter of measuring the percentage of dd-cfCDNA to great precision, our data remain consistent with the literature surrounding AlloSure and may differ slightly from the claims for the performance of Prospera. As suggested by Grskovic et al, this may be due to the study design of the Prospera validation study. In addition, results demonstrated AlloSure has a superior turnaround time from blood draw to patient result.

The concept of more being better is one we are all programmed to believe, yet this report supports the focused approach of AlloSure and confirms it is not outperformed by the more complex methodology used by Prospera and in fact may be more accurate in clinical interpretation and significantly faster to gain patient results. Further to this, both tests were drawn at the same time, both have similar workflows for ordering and both are dependent on courier-based service. In this regard, the superior AlloSure TAT makes it much more accessible in terms of making clinical decisions that guide patient care.

Assessing the overall performance of both, the AUC of the receiver operator curves were not statistically different. This is important, as diagnostic differences between assays would need very large numbers to be able to differentiate, yet the clinically relevant implications show the published data supporting AlloSure seems more robust, with more evidence based clinical utility.

Prospera missed 3 cases of TCMR1A using the 1% threshold which were detected by AlloSure using its published guidance of 0.5%. At current diagnostic cutoffs, there was no significant difference in sensitivity,
specificity, negative predictive value, or positive predictive value of AlloSure vs. Prospera in detecting rejection. Although not statistically significant; the clinical interpretation of TCMR1A is critical to clinical management of patients. The data published to guide interpretation of TCMR1A is much more robust for AlloSure and is consistent with our experience.9

This series has limitations due to an underpowered sample size, pilot experience and single time point used in each patient, which was done to preserve patients from additional and unnecessary blood work. A larger prospective, multi-center study is needed to validate these findings; however, these results suggest there may be little to gain. It is evident as more data is generated, that cross walking published dd-cfDNA data across different platforms may be ineffective and that although dd-cfDNA assays are similar they are not the same. This is increasingly important when managing patient populations, to allow consistent comparison using a single assay.

Considering growing evidence, re-evaluation of protocols to include dd-cfDNA monitoring has a place to support patients as part of their post-transplant surveillance. However, with the wide adoption of dd-cfDNA and the potential for further assays entering the field, a clear understanding of the technology and evaluation of clinical validation data supports the importance to remain consistent to a single platform.

More importantly, the published supporting evidence, use of real-life data, and the need for rapid turnaround to guide patient management is critical when considering clinical adoption.

Conclusions
Both dd-cfDNA platforms are useful and provide important adjuncts in the transplant clinician’s armamentarium. We believe that the use of dd-cfDNA monitoring will continue to expand clinically and that its future accuracy and precision will increase, but this needs to be supported by extensive clinical evidence and data when considering its implementation into a clinical program.

Disclosures
J.K. Melancon is on the Speaker Bureau for both CareDx and Natera and reports personal fees from Natera Inc and from CareDx Inc outside the submitted work. M.J. Lerman is a consultant for CareDx and reports personal fees in the form of honoraria from CareDx during the conduct of the study and outside the submitted work. The remaining author has nothing to disclose.

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Author Contributions
J Melancon: Conceptualization; Data curation; Formal analysis; Investigation; Writing - original draft; Writing - review and editing
A Khalil: Conceptualization; Data curation; Investigation; Validation
Supplemental Data

Supplement Table 1. Patient Cohort Demographics

References
Table 1. Side-by-side characteristics of AlloSure vs. Prospera

<table>
<thead>
<tr>
<th><strong>Characteristic</strong></th>
<th><strong>AlloSure</strong></th>
<th><strong>Prospera</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit of Detection</td>
<td>0.12%</td>
<td>0.15%</td>
</tr>
<tr>
<td>Claimed Sensitivity for TCMR</td>
<td>RCV above 0.5%⁹</td>
<td>100% at 1%⁷</td>
</tr>
<tr>
<td>Claimed AUC for all rejection</td>
<td>77%</td>
<td>87%</td>
</tr>
<tr>
<td>Number of SNPs</td>
<td>405</td>
<td>13962</td>
</tr>
<tr>
<td>Precision (CV)</td>
<td>4.5% and 7.7% for samples above and below 2% dd-cfDNA respectively⁵</td>
<td>4.29%⁶</td>
</tr>
<tr>
<td>Lowest input material needed</td>
<td>3ng⁵</td>
<td>15ng⁶</td>
</tr>
<tr>
<td>Blood Tubes Needed</td>
<td>1</td>
<td>2</td>
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</table>
Table 2. Side-by-side performance characteristics between AlloSure and Prospera for all rejection at 1% threshold. NPV (negative predictive value); PPV (positive predictive value).

<table>
<thead>
<tr>
<th>Diagnostic Category</th>
<th>AlloSure</th>
<th>Prospera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity [95% Exact CI]</td>
<td>0.4545 [0.1675, 0.7662]</td>
<td>0.5455 [0.2338, 0.8325]</td>
</tr>
<tr>
<td>Specificity [95% Exact CI]</td>
<td>0.8462 [0.5455, 0.9808]</td>
<td>0.6923 [0.3857, 0.9091]</td>
</tr>
<tr>
<td>PPV [95% Exact CI]</td>
<td>0.7143 [0.2904, 0.9633]</td>
<td>0.6000 [0.2624, 0.8784]</td>
</tr>
<tr>
<td>NPV [95% Exact CI]</td>
<td>0.6471 [0.3833, 0.8579]</td>
<td>0.6429 [0.3514, 0.8724]</td>
</tr>
</tbody>
</table>

Table 3A. Side-by-side performance characteristics between AlloSure and Prospera for cellular rejection at 0.5% threshold. NPV (negative predictive value); PPV (positive predictive value).

<table>
<thead>
<tr>
<th>Diagnostic Category</th>
<th>AlloSure</th>
<th>Prospera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity [95% Exact CI]</td>
<td>0.7778 [0.3999, 0.9719]</td>
<td>0.5556 [0.2120, 0.8630]</td>
</tr>
<tr>
<td>Specificity [95% Exact CI]</td>
<td>0.5333 [0.2659, 0.7873]</td>
<td>0.5333 [0.2659, 0.7873]</td>
</tr>
<tr>
<td>PPV [95% Exact CI]</td>
<td>0.5000 [0.2304, 0.7696]</td>
<td>0.4167 [0.1517, 0.7233]</td>
</tr>
<tr>
<td>NPV [95% Exact CI]</td>
<td>0.8000 [0.4439, 0.9748]</td>
<td>0.6667 [0.3489, 0.9008]</td>
</tr>
</tbody>
</table>

Table 3B. Side-by-side performance characteristics between AlloSure and Prospera for cellular rejection at 1.0% threshold. NPV (negative predictive value); PPV (positive predictive value).

<table>
<thead>
<tr>
<th>Diagnostic Category</th>
<th>AlloSure</th>
<th>Prospera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity [95% Exact CI]</td>
<td>0.4444 [0.1370, 0.7880]</td>
<td>0.4444 [0.1370, 0.7880]</td>
</tr>
<tr>
<td>Specificity [95% Exact CI]</td>
<td>0.8000 [0.5191, 0.9567]</td>
<td>0.6000 [0.3229, 0.8366]</td>
</tr>
<tr>
<td>PPV [95% Exact CI]</td>
<td>0.5714 [0.1841, 0.9010]</td>
<td>0.4000 [0.1216, 0.7376]</td>
</tr>
<tr>
<td>NPV [95% Exact CI]</td>
<td>0.7059 [0.4404, 0.8969]</td>
<td>0.6429 [0.3514, 0.8724]</td>
</tr>
</tbody>
</table>
**Table 4.** Side-by-side performance characteristics between AlloSure and Prospera – Area Under the ReceiverOperating Characteristic Curve (AUC ROC)

<table>
<thead>
<tr>
<th>Diagnostic Category</th>
<th>AlloSure</th>
<th>Prospera</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC ROC [95% CI]</td>
<td>0.7343 [0.5215, 0.9471]</td>
<td>0.7483 [0.5429, 0.9536]</td>
</tr>
</tbody>
</table>

**Figure 1.** Workflow of patients investigated with AlloSure and Prospera under surveillance or for-cause dd-cfDNA testing.
**Figure 2.** Three-part panel showing comparison of the difference in dd-cfDNA % across all sample paired dd-cfDNA percentages stratified by result (A) 0% to 1% (B) 0% to 20%, and (C) biopsy occurrence (excludes 1 sample above the limit of quantification)
Figure 3. Two-part panel showing the turnaround time and cumulative distribution of the Difference (AlloSure – Prospera) in Turnaround Time (TAT) between the assays