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The Impact of Sample Collection Order on the Diagnostic Performance of Metagenomic Deep Sequencing for Infectious Keratitis

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Abstract

Purpose: To evaluate the impact of sample collection order on the diagnostic yield of metagenomic deep sequencing (MDS) for determining the causative pathogen in infectious keratitis.

Methods: We performed a cross sectional diagnostic test evaluation among subjects with infectious keratitis at Aravind Eye Hospital in Madurai, India. All subjects underwent corneal scrapings of the affected eye to obtain KOH smear, Gram stain, bacterial culture, and fungal culture, in this order. The order of MDS specimen collection relative to smear and culture samples was randomized and served as the primary predictor. Outcomes included the normalized copy number of pathogenic RNA detected by MDS, the proportion of MDS samples which were diagnostic, and agreement of MDS results with cultures.

Results: MDS samples from 46 subjects with corneal ulcers were evaluated. MDS was positive in 33 subjects (76%) and had 74% overall agreement with culture results. There was no association between order of MDS sample collection and normalized copy number of genetic material detected (P=0.62) or the likelihood of MDS positivity (P=0.46). However, the likelihood of agreement between MDS and cultures decreased when MDS corneal swabs were collected after other diagnostic corneal scrapings (P=0.05).

Conclusions: The overall yield of MDS for detecting the cause of infectious keratitis was not affected by sample collection order. However, diagnostic agreement between MDS and cultures decreased when MDS samples were collected after other specimens. Additional investigation is warranted to determine whether this represents increased sensitivity of MDS compared to cultures or higher susceptibility to contaminants.

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Conflicts of interest: none

Keywords

Metagenomic deep sequencing; infectious keratitis; corneal ulcer

INTRODUCTION

Corneal opacification is the 5th leading cause of blindness worldwide, with the majority of cases attributable to infection.[1–4] Directed antimicrobial treatment improves visual outcomes, but gold standard culture methods fail to identify the causative pathogen in 40–60% of cases.[5,6] Thus the development and implementation of adjunctive diagnostic methods for infectious keratitis may have a significant impact on global blindness. Metagenomic deep sequencing (MDS) is an unbiased, hypothesis-free diagnostic testing method capable of detecting both common and unusual pathogens with minimal sample material required. In addition to identifying causative pathogens, MDS may have applications in evaluating antibiotic susceptibilities, host response to infection, and the epidemiology of infectious diseases. The utility of MDS as a diagnostic tool for infectious disease has been demonstrated in uveitis, conjunctivitis, scleritis, and a number of systemic conditions.[7–11] With respect to infectious keratitis, a small preliminary study demonstrated high concordance between MDS and conventional culture results in a variety of cases including bacterial, fungal, viral, and parasitic keratitis.[11]

A subsequent study of MDS as a diagnostic tool for infectious keratitis evaluated 46 undifferentiated corneal ulcers presenting to Aravind Eye Hospital in Madurai, India, and determined that metagenomic RNA deep sequencing demonstrated higher sensitivity (100%) and specificity (97%) than DNA sequencing and traditional microbiologic methods such as potassium hydroxide smear, Gram stain, and cultures using latent class analysis.[12] These results were particularly compelling in the setting of South India where corneal ulcers typically have very high infectious load, a feature that would be expected to favor traditional culture methods which typically require more pathogenic material compared to nucleic acid amplification techniques.[13]

This early evidence suggests there may be a role for MDS as an adjunctive diagnostic modality for infectious keratitis. However, its implementation does not obviate the need to obtain traditional biological stains and cultures, thus several specimens must be collected from the ulcer at a single visit. The impact of repeated sampling from a corneal ulcer on the diagnostic yield of MDS is unknown. Theoretically, yield may be lower when MDS is collected after other specimens due to the potential removal of pathogens by prior scrapings. Alternatively, yield may be increased if initial scrapings predominantly remove necrotic debris, allowing access to the underlying causative organism. Determining the impact of sample collection order on the diagnostic yield of MDS may allow a more strategic approach to specimen collection, maximizing the likelihood of pathogenic organism detection. In this study we performed a secondary analysis of the 46 corneal ulcers evaluated at Aravind to determine whether the diagnostic yield of MDS in infectious keratitis is affected by the order of MDS sample collection relative to other microbiologic specimens.[12]

METHODS

Participants

Eligible participants included all subjects presenting to the Aravind Eye Hospital in Madurai, India with a clinical diagnosis of infectious keratitis, regardless of severity or etiology. Informed written consent was obtained from all participants. Institutional Review Board approval was obtained at Aravind Eye Hospital and the University of California San Francisco. All aspects of the study adhered to the tenets of the Declaration of Helsinki.

Specimen Collection

After instillation of topical anesthesia, corneal scrapings were obtained for routine microbiologic testing using a heat-sterilized Kimura spatula. These specimens were processed in pre-specified order for all patients: the first corneal scraping was used to perform potassium hydroxide (KOH) wet mount, the second for Gram stain, the third for bacterial culture on sheep's blood agar, and the fourth for fungal culture using potato flake agar.

The MDS specimen was obtained from the site of ulceration using a sterile polyester tipped applicator (Puritan, Guilford, ME) and placed immediately in DNA/RNA-Shield (Zymo Research, Irvine, CA). A second swab was obtained from the inferior fornix of the unaffected, contralateral eye and stored in similar fashion. Care was taken to avoid contamination of MDS specimens by wearing a mask and gloves during specimen collection and avoiding over-handling the specimen container. The order of MDS specimen collection relative to the four corneal scrapings described above was determined by a smartphone random number generator, with options being any integer from 1 to 5. For example, if the MDS sample order was specified as "3", specimens would be collected from the ulcer in the following order: first KOH, then Gram stain, then MDS, then bacterial culture, then fungal culture. MDS specimens were stored at −80C until shipped on dry ice to the Proctor Foundation/University of California San Francisco. Samples were subsequently stored at −80C until processed for sequencing.

Interpretation of Stain and Culture Results

Biological stains (KOH smear and Gram stain) and cultures were interpreted by microbiology staff at Aravind according to standard procedure. Any conflicting results between stains and cultures required adjudication before assigning the final determination according to standard microbiologic methods. We determined that in cases with Gram stain results demonstrating features consistent with common commensal organisms on the ocular surface (for example, Gram positive bacilli) but with negative culture results, this would be interpreted as contaminant and the specimen would be read as negative according to traditional microbiologic methods.[14] Cases with fungal elements identified on KOH smear were identified as positive for fungal infection even in the setting of negative fungal culture results. This is due to the relatively low sensitivity of fungal cultures, and has been previously used as the standard for inclusion in several randomized clinical trials for fungal keratitis.[15–17]

MDS Processing

All laboratory personnel were masked to the identity of the samples.

Metagenomic deep sequencing was performed as previously described[7]. Briefly, total RNA was extracted from the Zymo media containing the swabs and reverse transcribed to double-stranded cDNA. The cDNA was converted to Illumina libraries and amplified with 16 PCR cycles. For DNA-seq, total DNA was extracted and fragmented prior to Illumina library processing. The sample was sequenced on the Illumina HiSeq 4000 or NovaSeq using 150-nucleotide (nt) paired-end sequencing. Analysis of sequenced data was made utilizing a computational pipeline developed in-house to classify sequencing reads and identify potential pathogens by aligning to the NCBI nt database. Because the ocular surface is an exposed site, for this study, the taxa identified from the control contralateral conjunctiva were identified and bioinformatically subtracted prior to final analysis. In cases where the suspected pathogen was common to all conjunctiva flora, a water control from the same sequencing run was used as background subtraction. The organism was identified as positive by MDS if it was known to cause ocular infection and if it represented the most abundant reads after human and contralateral conjunctival “background” or water subtraction. Interpretation of MDS results was performed in a masked fashion with respect to smear and culture results.

Statistical Analysis

The primary outcome of interest was the diagnostic yield of MDS for potential pathogens, which is challenging to quantify in the absence of a true gold standard diagnosis. To address this we utilized three surrogate measures to approximate diagnostic yield: 1) log rM (reads per million reads) 2) Proportion of positive MDS results; and 3) Agreement between MDS and traditional microbiologic methods. We considered MDS copy number (log rM) to be the primary outcome, with MDS positivity and agreement between MDS and culture results considered secondary outcomes. A sample size of 46 provided 80% power to detect an 18% difference in log rM at an alpha of 0.05. The primary predictor was the order of MDS specimen collection relative to other microbiologic samples obtained. Covariates included whether the ulcer had been pre-treated with antimicrobial agents prior to specimen collection and the severity of infection represented by the subsequent development of perforation within 4 weeks following specimen collection. MDS copy number was assessed using bivariate comparisons and multivariate linear regression. The proportion of positive MDS results and percent agreement between MDS and traditional microbiologic methods were evaluated using bivariate comparisons and multivariate logistic regression. All statistical analyses were performed using R version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

46 participants with unilateral corneal ulcers were identified. Demographic characteristics are described in Table 1. MDS was the 1st sample collected in eight cases, the 2nd sample collected in eight cases, the 3rd sample collected in 14 cases, the 4th sample collected in eight cases, and the final sample collected in eight cases. MDS detected a causative

pathogen in 34 cases (74%). According to MDS, 20 ulcers (59%) were fungal and 14 (41%) were bacterial. Standard microbiologic methods were positive in 31 cases (67%); 24 ulcers (77%) were deemed fungal and 7 (23%) bacterial. MDS had 74% overall agreement with standard methods (kappa 0.39; P=0.009). In 7 cases, MDS detected a pathogen and standard microbiologic evaluations did not. The pathogens detected by MDS in these cases were *Pseudomonas pseudoalcaligenes, Bacillus* genus bacteria, *Aspergillus oryzae, Burkholderia cepacia, Aeromonas hydrophila, Streptococcus pneumoniae,* and *Moraxella osloensis.* In 4 cases, standard microbiology methods detected a pathogen while MDS did not. The pathogens detected by standard methods in these cases were *Streptococcus viridans* and 3 unspecified filamentous fungal species in the remaining cases (fungal elements identified on KOH smear but fungal cultures negative).

There was no association between MDS sampling order and copy number of pathogenic organisms detected (multivariate linear regression; P=0.62) or between MDS sampling order and the likelihood of a positive MDS result (logistic regression, odds ratio=1.21 [95% confidence interval 0.74–2.02]; P=0.46) (Figure 1; Table 2). However, there was a statistically significant association between MDS sampling order and the level of agreement between MDS results and standard microbiologic results (logistic regression, odds ratio=0.57 [95% confidence interval 0.31–0.97]; P=0.05). Specifically, MDS specimens collected 5th in the sampling order were 43% less likely to agree with standard microbiologic results compared to MDS specimens collected 1st. Many of these disagreements appeared to be due to MDS detecting a pathogen while standard microbiologic methods did not. MDS specimens obtained later in the sampling order were more likely to be positive with negative standard microbiologic results compared to MDS specimens obtained earlier in the sampling order, but this association was not statistically significant (logistic regression, odds ratio=1.91 [95% confidence interval 0.99–4.30]; P=0.08) (Figure 1). Pre-treatment with antimicrobial agents and subsequent development of perforation were not statistically significant predictors of any outcome examined and thus were not included in any of the final regression models.

DISCUSSION

In this study we assessed the impact of the order of microbiologic sample collection on the diagnostic yield of MDS for pathogen detection in infectious keratitis. The key findings are: 1) The overall diagnostic yield of MDS was not affected by whether the MDS corneal specimen was collected before or after other corneal scrapings for conventional microbiologic diagnostics; and 2) MDS was more likely to disagree with standard microbiologic results when the MDS specimen was collected after other microbiologic samples. These results may inform strategic prioritization of sample collection when multiple specimens are collected contemporaneously, and inform future studies to better characterize the relationship between sampling order and diagnostic yield.

In theory, proper microbiologic specimen collection should strive to achieve a balance between limiting contaminant and non-diagnostic debris while maximizing the number of pathogenic organisms in a sample. To our knowledge, no objective evaluations of the impact of microbiologic specimen collection order on diagnostic yield exist in the

literature. However, there is a prevailing presumption that the balance between diagnostic and non-diagnostic material contained within a specimen may change with serial sampling and has informed practices such as “clean catch” urine specimen collection and brush debridement of the cervix prior to obtaining a pap smear. Nonetheless, objective evidence has demonstrated no change in the likelihood of urine specimen contamination with clean catch technique compared to standard specimen collection.[18–21] No direct comparisons have evaluated the diagnostic yield of the first and second cervical swab for HPV detection, but self-collected vaginal swabs with no debridement have been shown to be non-inferior to the traditional colposcopic method using debridement of the cervical os prior to collection of a sample for HPV testing.[22] With respect to infectious keratitis, several studies have compared the diagnostic yield of single vs multiple corneal specimens, but none have evaluated the impact of sample collection order when obtaining multiple samples.[23–27] Thus our finding that the overall yield of MDS was not impacted by its position in the sample collection order is novel. This may be attributable to the exquisitely high sensitivity of this test and consequently minimal required sample in order to obtain a result.

However, the high sensitivity of MDS may increase the risk of detecting non-pathogenic organisms compared to traditional microbiologic methods. The ocular surface is frequently populated with commensal organisms which can be confused for the causative pathogen when infection is present. This emphasizes the importance of identifying the microbial genomic material of the contralateral unaffected eye (in unilateral cases) and the rationale for a water control subtraction when infection with a commonly encountered commensal organism (e.g. *Staphylococcus aureus*) is the suspected pathogen. Further, the assumption that the ocular surface microbiome is equivalent between the two eyes at baseline has not been tested. In addition to commensal organisms on the ocular surface, the very high sensitivity of MDS makes it susceptible to potential contamination from the environment. In this study we found that MDS specimens collected later in the sampling order were more likely to disagree with traditional microbiologic methods, often because MDS detected a potential pathogen when traditional methods did not. This may support the notion that MDS is erroneously detecting commensal organisms particularly when most or all of the pathogenic organism has been removed by preceding sample collection. However, the organisms detected by MDS in these cases were *Aspergillus oryzae, Aeromonas hydrophila, Streptococcus pneumoniae, Bacillus,* and *Moraxella osloensis*, all of which have been reported to cause keratitis in humans.[28–30] It is possible that MDS was actually detecting the pathogenic organism in these cases when culture failed to do so, particularly considering the relatively low sensitivity of cultures at baseline, prior use of antimicrobial agents in many cases, and the very high specificity (97%) of MDS for infectious keratitis in prior studies indicating a low likelihood of false positive results.[5,6,12] However, this would not explain why this difference between MDS and standard methods occurred more frequently when MDS was collected later in the sampling order. Larger multicenter studies comparing MDS to traditional methods for the etiologic diagnosis of infectious keratitis and comparisons of the yield of each diagnostic modality when obtained consecutively from the same corneal ulcer are warranted to address these questions.

This study has several limitations. First, we recommend caution prior to extrapolating these results outside of India due to potential geographic differences in the etiology, severity, and

pathogenic load of infectious keratitis.[13,31] Second, it should be emphasized that evaluations of the accuracy of a diagnostic test are limited in the absence of a true gold standard, which does not exist for infectious keratitis. This was the rationale for evaluating three distinct surrogates of diagnostic yield in this study. Additionally, larger studies comparing the accuracy of MDS and standard microbiologic methods are needed to definitively establish the most accurate diagnostic test currently available. Third, this study may have been underpowered to detect minute differences in the mean copy number identified by MDS due to small sample size. Fourth, the quality of MDS output critically depends on sample processing methodology, bioinformatics approach, and the available or annotated reference database for pathogen detection. Fifth, the duration of antibiotic pretreatment was not known for subjects included in this study. Additionally, topical anesthetic was administered prior to specimen collection, which may affect organism viability. However, because MDS collection order was randomized, these factors would not be expected to introduce any differential bias into the results of this study. However, differences in the duration of antibiotic pre-treatment may have potentially introduced non-differential bias, which would have biased the results toward the null hypothesis and further reduced the power of this study. This could explain lack of an association between MDS specimen collection order and overall diagnostic yield, but could not have erroneously generated the positive associations identified. Finally, it should be noted the cost of MDS is typically much higher than standard culture methods or PCR, which may prohibit widespread adoption. However, this cost is expected to decrease over time.

In conclusion, the overall diagnostic yield of MDS for infectious keratitis was not affected by the order of microbiologic sample collection in this study. However, MDS samples collected later in the sampling order tended to disagree with the results of standard microbiologic specimens. It is unclear whether this may indicate that MDS samples obtained later in the specimen collection order are more sensitive to contaminants than biologic stains and cultures, or that MDS is able to detect pathogens not otherwise identified by standard microbiologic methods due to differences in threshold sensitivity for organism detection. Future studies are needed to investigate this relationship.

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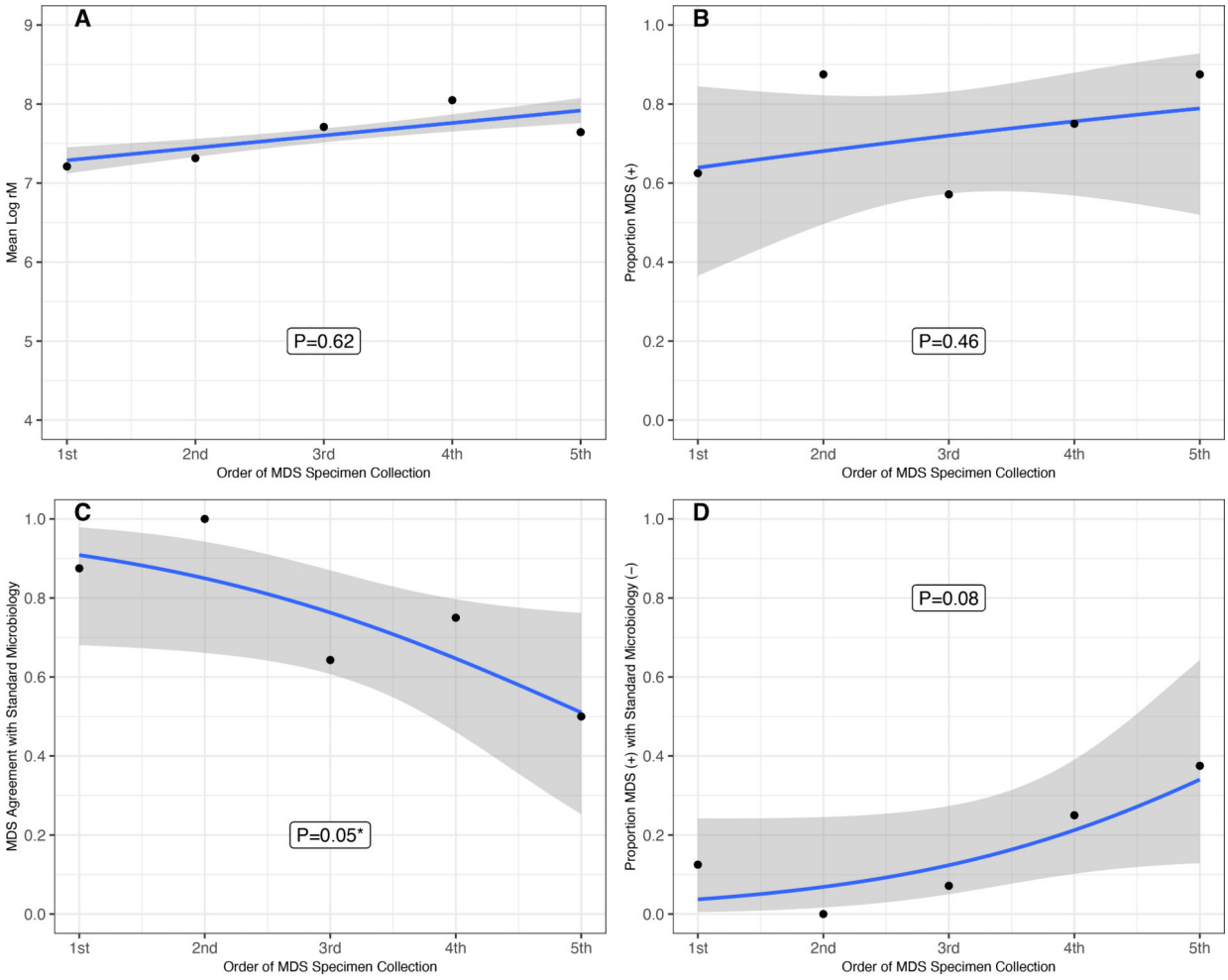

Figure 1:

Relationship between the order of MDS specimen collection and diagnostic yield with respect to log rM (A), proportion of positive MDS results (B), agreement between MDS and standard microbiologic methods (C), and proportion of positive MDS results in the setting of negative standard microbiologic results (D). Abbreviations: rM (reads per million reads); MDS, metagenomic deep sequencing.

Table 1:

Demographic Characteristics of 46 Participants with Corneal Ulcers at the Aravind Eye Hospital

	mean±SD years(range)	n(%)
Age	55±14(13–86)	-
Sex		
Male	-	31(67)
Female	-	15(33)
Prior use of antimicrobial therapy	-	25(54)
Subsequent perforation[1]	-	9(20)
Infectious etiology[2]		
Bacterial	-	7(15)
Fungal	-	24(52)
None Detected	-	15(33)

[1] Within four weeks of presentation

[2] According to standard microbiology methods

Table 2:

Four regression models evaluating the impact of sample collection order on the diagnostic yield of metagenomic deep sequencing samples from 46 corneal ulcers.

Regression Models Evaluating the Effect of MDS[1] Sample Collection Order on Diagnostic Yield Among 46 Corneal Ulcers					
	coefficient	**SE[2]**	**Z score**	**OR[3] (95% CI[4])**	**P value**
Linear Regression with MDS Copy Number (log rM) as Outcome					
(intercept)	7.13				
MDS specimen collection order	0.16	0.32	0.50	N/A	0.62
Logistic Regression with MDS Positivity as Outcome					
(intercept)	0.38				
MDS specimen collection order	0.19	0.25	0.74	1.20 (0.74–2.02)	0.46
Logistic Regression with Agreement Between MDS and Cultures as Outcome					
(intercept)	2.86				
MDS specimen collection order	−0.56	0.29	−1.96	0.57 (0.31–0.97)	0.05
Logistic Regression with MDS Positivity When Cultures Were Negative as Outcome					
(intercept)	−3.90				
MDS specimen collection order	0.65	0.37	1.77	1.91 (0.99–4.30)	0.08

*Covariates including the duration of pre-treatment with antimicrobials and severity of ulcer were not significant predictors of any of the above outcomes and thus were not included in the final regression models

[1]MDS = metagenomic deep sequencing

[2]SE = standard error

[3]OR = odds ratio

[4]CI = confidence interval