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Unbiased Pathogen Detection and Host Gene Profiling for Conjunctivitis

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Abstract

Purpose: The etiology of conjunctivitis is often misdiagnosed. An ideal diagnostic test would identify all possible infectious causes. In this study, we apply unbiased metagenomic RNA deep sequencing (MDS) to identify pathogens causing conjunctivitis.

Design: Molecular study of prospectively collected conjunctival swabs from patients with presumed infectious conjunctivitis.

Participants: Patients with presumed acute infectious conjunctivitis.

Methods: Conjunctival swabs were collected from patients presenting with acute conjunctivitis. Swabs were processed for MDS. Pathogens were identified using a rapid computational pipeline to analyze the non-host sequences obtained from MDS. Differential gene expression analysis was performed to evaluate for host transcriptome signatures for infectious types. Clinical samples were de-identified and laboratory personnel handling the samples and interpreting the data were masked.

Main Outcome Measures: Pathogens and differential transcripts identified by MDS.

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Results: MDS detected pathogens in 86% (12/14) of the patients tested. Swabs from 10 of 14 patients were positive for human adenovirus (HAdV) while swabs from 2 of 14 patients were positive for *Vittaforma corneae* (a parasitic fungal species of the microsporidia group). Samples positive for HAdV by RNA-seq were independently verified in a CLIA-certified laboratory. Directed pathogen-PCR confirmed the presence of *V. corneae* genome in the samples positive by RNA-seq. Local host transcriptome analysis identified 12 differentially expressed genes that provided distinct expression signatures for patients infected with HAdV compared to *V. corneae*.

Conclusions: MDS can reliably detect and quantify common and rare pathogens causing conjunctivitis, and identify strains. The unbiased nature of metagenomic RNA deep sequencing allowed an expanded scope of pathogen detection, including fungal species not commonly associated with acute conjunctivitis. In addition, the identification of infection type-specific local host transcriptome signatures may allow for pathogen detection even when the pathogen load is too low for direct identification.

INTRODUCTION

Infectious conjunctivitis, both acute and epidemic, is a common diagnosis worldwide. Although diagnosis is mainly dependent on clinical exam, clinical manifestations are non-specific and have a low accuracy in predicting the etiologic agent.[1] For example, profuse tearing, swelling and irritation is classically considered to be viral in origin, but up to 50% of viral conjunctivitis are misdiagnosed.[2] While human adenovirus (HAdV) is the most common etiology for acute infectious conjunctivitis, other viruses, bacteria, and fungi remain important causes.[3–6]

Commonly used diagnostics include cultures, antigen detection, and pathogen-directed PCRs.[7] Cultures, however, have low sensitivity because of numerous factors, *i.e.* media and incubation temperatures, that can interfere with successful growth of microbes in a laboratory environment. Antigen detection and pathogen-directed PCRs have improved sensitivity, but require *a priori* targets. Hence, the limitation of these molecular assays is that other pathogens may have been present but entirely missed. The emergence of new unbiased diagnostic tools, such as metagenomic RNA deep sequencing (MDS), may allow for a more comprehensive characterization of disease. MDS not only can detect pathogen but has the potential to query the host immune response to the infection.

In this study, we applied MDS to identify pathogenic agents and the local host transcriptome signatures in patients with presumed acute infectious conjunctivitis.

METHODS

Patient Selection.

Conjunctival swabs were obtained from patients who presented to the Aravind Eye Hospital in India from January 2017 to January 2018. Inclusion criteria were: 1) acute conjunctivitis (new and sudden onset of tearing, redness, and ocular irritation within the past 14 days), 2) lids with P2 (at least 50% of the underlying normal vessels are hazy but not obscured) and higher *or* F1 (5 or fewer follicles) and higher.[8] Exclusion criteria were: 1) presumed allergic conjunctivitis, and 2) presumed conjunctivitis medicamentosa. This study adhered to the

tenets of the Declaration of Helsinki. The Institutional Review Board of the University of California, San Francisco (UCSF) and the Aravind Eye Hospital approved the study. Informed written consent was obtained from all patients.

Samples.

The lower fornix of the affected eye was swept 3 times using a sterile polyester tipped applicator (Puritan). The swab was immediately placed in DNA/RNA-Shield (Zymo Research) to preserve the integrity of the nucleic acids in the sample, and transferred to a −80°C freezer for storage. Samples were subsequently shipped to the Proctor Foundation/ UCSF on dry ice and stored at −80°C until tested.

Library Preparation, Sequencing, and Bioinformatics.

Total RNA was extracted from the conjunctival samples using the ZR-Duet extraction kit (Zymo Research) per manufacturer's instructions. Sequencing libraries were prepared and sequenced as previously described.[9–11] Sequencing data were analyzed using a rapid, in-house computational pipeline to classify MDS reads and identify potential pathogens by comparison to the entire NCBI nucleotide (nt) reference database.[9, 12–14] Given the small sample size, we implemented a conservative and simple approach to avoid over interpretation of the sequencing data. First, the water control was used to identify environmental and laboratory contaminants. The list of organisms detected in the water control was then used to background subtract from the list of organisms detected in the tested patient samples. The remaining organisms were considered to be credible "hits" warranting further confirmatory testing if the following criteria were met: (1) the organism had >10 non-redundant, mapped read pairs at the species level based on nt alignment, and (2) the organism was known to be associated with ocular infection. edgR was used to perform transcriptome analysis.[15] Genes with a false-discovery rate (FDR) < 0.01 were considered to be significant. DAVID (Database for Annotation, Visualization and Integrated Discovery) and g:Profiler web-based functional gene profiling pipelines were used to evaluate for pathway enrichment.[16–18]

Confirmatory Testing.

Samples that underwent MDS were also subjected to HAdV-directed PCR at the CLIA-certified Stanford Clinical Virology Laboratory. This real-time quantitative PCR (qPCR) detects all HAdV serotypes. The detection of *V. corneae* was performed with published primers[3] using the One-Step RT PCR with Platinum Taq master mix (Thermo Fisher Scientific). The product was Sanger sequenced (Elim Biopharm) and verified with the Basic Local Alignment Search Tool (BLAST).

RESULTS

MDS was used to determine the cause of conjunctivitis in 14 patients. The clinical characteristics of these patients are shown in Table 1. Samples were sequenced to an average depth of 13,112,368 (ranging from 9,837,977 to 17,373,423) reads/sample. Of those reads, 96.5% (SD=0.6%) were non-host reads and were filtered prior to analysis for pathogen detection (Supplementary Figure 1, available at www.aaojournal.org). Of the 14 samples

tested, 12 (86%) samples were pathogen positive. HAdV DNA was detected in 10 (83%) of the positive samples by MDS (Figure 1A); and confirmed with HAdV-directed qPCR (Figure 1B). The number of HAdV reads detected with MDS spanned 4 orders of magnitude and strongly correlated with the virus load determined by HAdV qPCR ($r = 0.93$, $P = 0.0002$, Spearman's correlation test, Figure 1B). These results demonstrate the wide range of concentrations detectable by MDS and its ability to provide semi-quantitation of the viral burden in conjunctival samples. Sequence analysis indicated the presence of HAdV-D8, a genotype commonly associated with conjunctivitis, in all samples.

Vittaforma corneae was detected in 2 (14%) conjunctival samples by MDS (Figure 1C–D). These results were confirmed with directed PCR. Both patients were immunocompetent. While the predominant clinical finding was unilateral conjunctivitis, both patients had corneal involvement.

An additional advantage of MDS is the ability to concurrently interrogate host sequence information to identify transcriptome signatures for infection types. As a proof-of-concept, we compared the samples positive for HAdV to those positive for *V. corneae*. Of all host transcripts interrogated, we identified 12 differentially expressed genes that distinguished HAdV vs *V. corneae* infection (Figure 2). Five genes were differentially upregulated and 7 genes were downregulated in the HAdV infected group compared to *V. corneae* infected group (Table 1, available at www.aaojournal.org). Similarly, we detected a transcriptome signature for samples negative for pathogens when compared to those positive for HAdV (Table 2, available at www.aaojournal.org). These transcripts are enriched for chemokine signaling and regulation of cell motility and migration pathways.[16–18]

DISCUSSION

In this study, we found that MDS can reliably detect causative agents of conjunctivitis. 86% of the conjunctival samples tested were positive for a pathogen. While it was not surprising that HAdV infection was the predominant cause in the majority of cases, the identification of *Vittaforma corneae* infection in 14% of the cases highlighted the expanded potential of an unbiased diagnostic test.

V. corneae is an obligate, spore-forming, intracellular parasite that belongs to the phylum *Microspora*. It is a known cause of keratitis and keratoconjunctivitis in immunocompromised and immunocompetent patients.[5, 19, 20] Exposure to contaminated water, soil, foreign body, or trauma can be risk factors for *V. corneae* infection[5], although infection can occur without an identifiable risk factor, as was the case with both patients enrolled in this study. The majority of immunocompetent patients with *V. corneae* infection in the setting of keratoconjunctivitis have greyish-white, coarse, multifocal, and raised ("plaque-like") superficial punctate epithelial lesions. Both patients in this study had unilateral eye involvement, although bilaterality has been previously reported in ~4% of patients.[5] Clinical findings alone, therefore, may be misleading and may result in erroneous diagnosis. Patient #8 was clinically diagnosed with *V. corneae*, when the patient was positive for HAdV on molecular testing. While there is no effective treatment for viral conjunctivitis, antifungals can provide viable options for the treatment of microsporidial

keratoconjunctivitis.[5, 21] Further, confirmation of viral etiology can limit inappropriate antibiotic exposure.

A main benefit of MDS is its ability to interrogate all genomes in any clinical sample. In order to generate accurate pathogen identification, meticulous removal of human host sequences, constituting >95–99% of all sample sequences, is required. Here, in addition to pathogen detection, we queried the host transcription to determine if the local host immune response is different dependent on inciting pathogen. Indeed, we were able to identity a panel of candidate genes that could distinguish adenoviral infection from *V. corneae* infection. These genes have the potential to provide alternative molecular targets for the detection of these pathogens.

Limitations of this study include the small sample size and that all patients were recruited from a single eye center in India. Meaningful statistical differentiation of the clinical presentation and ocular findings for each infection type was not possible due to the small sample size. In addition, 64% of acute conjunctivitis patients did not return for a follow up appointment, hence precluding analysis of clinical outcome. Future studies would benefit from MDS analysis of samples collected from geographically diverse locations to comprehensively catalog the causes of conjunctivitis.

In summary, infectious conjunctivitis is heterogenous in etiology. MDS provides an unbiased approach to detect common and rare pathogens, determine pathogen load, provide viral typing, and identify host immune response. Local host transcriptomic signatures have the potential to improve diagnostics and provide new therapeutic targets for infections previously without effective treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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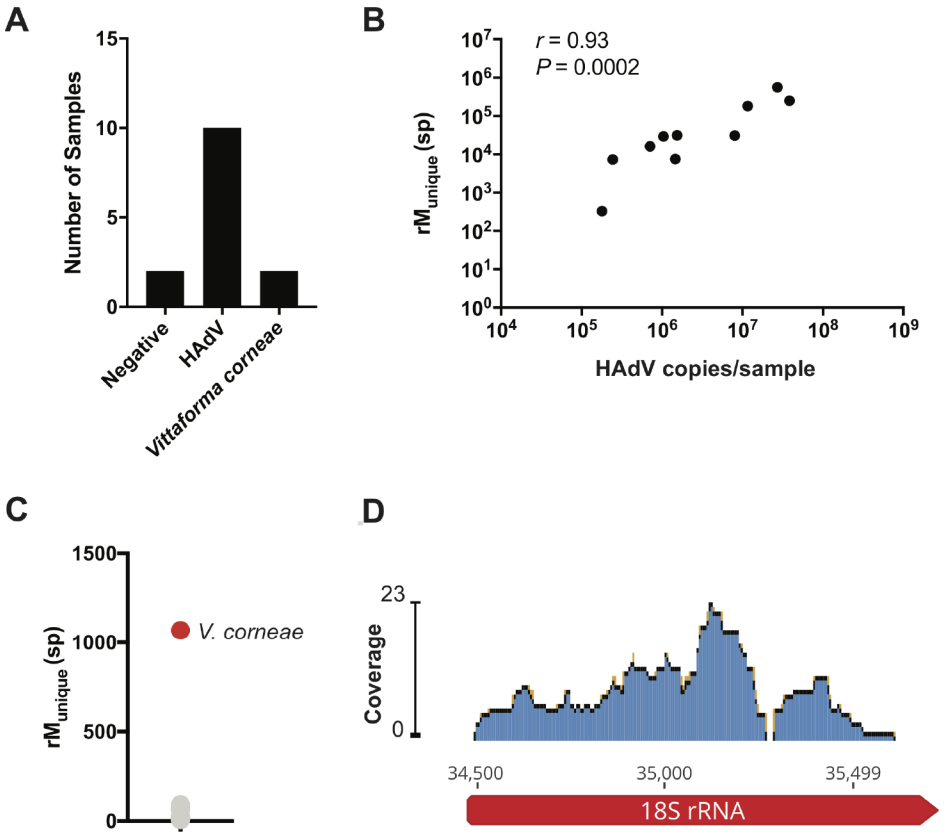

Figure 1.

Pathogens detected by metagenomic RNA sequencing (MDS). (A) Results of conjunctiva samples tested by MDS. (B) The correlation between viral copies as detected by HAdV quantitative PCR and normalized HAdV reads by MDS ($r = 0.93$, $P = 0.0002$, Spearman correlation test). (C) Reads aligning to *V. corneae* were the predominant non-host reads in both *V. corneae* positive samples. Sample from patient #11 is shown here. (D) Alignment of *V. corneae* reads to the *V. corneae* genome. Only the 18 S rRNA region is shown for clarity. Abbreviations: HAdV, human adenovirus; rM, reads per million reads.

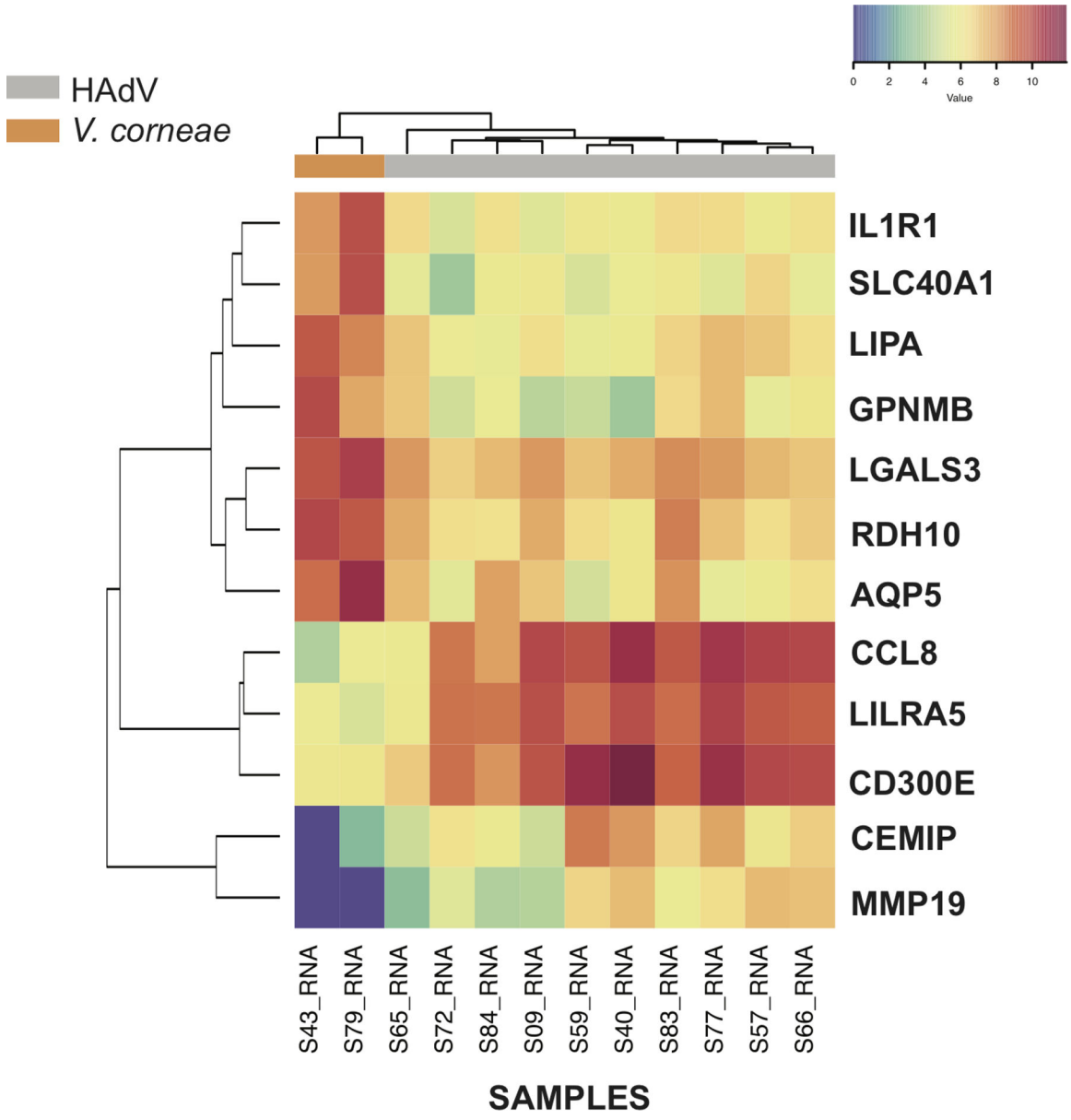

Figure 2.

Host transcriptional profiling distinguishes HAdV versus *V. corneae* infection. Normalized expression levels, arranged by unsupervised hierarchical clustering, reflecting over-expression (red) or under-expression (blue) of genes (rows) for each conjunctiva sample (columns). 12 differential expressed genes identified with FDR <0.01. Abbreviation: HAdV, human adenovirus; FDR, false discovery rate.

Table 1:

Demographics and Clinical Characteristics of Patients Enrolled

Abbreviations: HAdV, human adenovirus; *V. corneae*, *Vittaforma corneae*; OD, oculus dexter; OS, oculus sinister.

Patient #	Age	Gender	Eye Swabbed	Presenting Visual Acuity of Swabbed Eye	Presenting Clinical Details	Bilateral Involvement	Duration of Symptoms (Days)	Topical Medication Use/Name	Sequencing Results
1	48	F	OS	20/20	Tearing, Redness	No	3	Yes/Unknown	HAdV-D8
2	50	F	OD	20/20	Tearing, Redness	No	3	Yes/Unknown	HAdV-D8
3	70	M	OD	20/60	Irritation, Redness, Plaquelike superficial punctate keratitis	No	3	Yes/Ganciclovir	*V. corneae*
4	47	M	OD	20/30	Pain, Eyelid swelling, Redness	Yes	4	Yes/Ofloxacin, Ciproflox	HAdV-D8
5	40	F	OS	20/30	Irritation,Tearing, Eyelid swelling, Tearing	No	3	No	HAdV-D8
6	52	M	OD	20/200	Irritation, Redness, Tearing, Superficial punctate keratitis	No	7	Yes/Artificial tears	HAdV-D8
7	38	M	OD	20/20	Irritation, Tearing, Eyelid swelling	No	2	No	HAdV-D8
8	25	M	OD	20/40	Tearing, Redness	Yes	2	Yes/Unknown	HAdV-D8
9	26	M	OS	20/40	Irritation, Redness	Yes	3	Yes/Gatifloxacin, Ofloxacin	Negative
10	31	M	OS	20/40	Irritation, Redness, Tearing	Yes	4	Yes/Ofloxacin, Zoxan, Aurocol	HAdV-D8
11	41	F	OS	20/20	Pain, Eyelid swelling, Redness, Plaque-like superficial punctate keratitis	No	1	No	*V. corneae*
12	40	M	OS	20/30	Irritation, Eyelid swelling, Tearing	No	4	Yes/Unknown	HAdV-D8
13	42	M	OS	20/20	Redness, Irritation	Yes	5	No	HAdV-D8
14	22	M	OS	20/40	Tearing, Redness, Punctate epithelial erosions	No	2	Yes/Unknown	Negative