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1 **Title:** Illuminating Uveitis: Metagenomic Deep Sequencing Identifies Common and Rare Pathogens

2

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21 **SUMMARY**

22 **Background**

23 Ocular infections remain a major cause of blindness and morbidity worldwide. While prognosis is
24 dependent on the timing and accuracy of diagnosis, the etiology remains elusive in ~ 50% of
25 presumed infectious uveitis cases.[1,2] We aimed to determine if unbiased metagenomic deep
26 sequencing (MDS) can accurately detect pathogens in intraocular fluid samples of patients with
27 uveitis.

28

29 **Methods**

30 This is a proof-of-concept study, in which intraocular fluid samples were obtained from 5 subjects
31 with known diagnoses, and one subject with bilateral chronic uveitis without a known etiology.
32 Samples were subjected to MDS, and results were compared with conventional diagnostic tests.
33 Pathogens were identified using a rapid computational pipeline to analyze the non-host sequences
34 obtained from MDS.

35

36 **Findings**

37 Unbiased MDS of intraocular fluid produced results concordant with known diagnoses in subjects
38 with (n=4) and without (n=1) uveitis. Rubella virus (RV) was identified in one case of chronic
39 bilateral idiopathic uveitis. The subject's strain was most closely related to a German RV strain
40 isolated in 1992, one year before he developed a fever and rash while living in Germany.

41

42 **Interpretation**

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43 MDS can identify fungi, parasites, and DNA and RNA viruses in minute volumes of intraocular
44 fluid samples. The identification of chronic intraocular RV infection highlights the eye's role as a
45 long-term pathogen reservoir, which has implications for virus eradication and emerging global
46 epidemics.
47
48

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49 **INTRODUCTION**

50 Ocular infection is an important cause of ocular morbidity and blindness worldwide. However,
51 diagnosis is challenging due to the multitude of possible pathogens. Sensitivity of culture-based
52 assays ranges from 40-70%, and available molecular diagnostics target only a fraction of pathogens
53 known to cause ocular disease.[1-3] These limitations are exacerbated by: (1) the inability to collect
54 large intraocular fluid volumes given the eye's small and delicate anatomy, and (2) the difficulty
55 distinguishing clinically between infectious and non-infectious causes of ocular inflammation.
56

57 The urgency to develop better diagnostics for uveitis has been compounded by the recent cases of
58 persistent infection with Ebola virus[4], and possibly Zika virus.[5] These cases highlight the eye's role
59 as a potential reservoir for infectious agents with important public health consequences. It is
60 essential that more sensitive, unbiased, and comprehensive approaches are developed to efficiently
61 diagnose ocular infections.
62

63 Rapid advances in sequencing technology and bioinformatics have made metagenomics a fertile
64 area for developing clinical diagnostics.[6-8] We evaluated the utility of an hypothesis-free approach
65 to identify ocular infections by performing unbiased metagenomic deep sequencing (MDS) on total
66 RNA extracted from the intraocular fluid of subjects with inflammatory and non-inflammatory eye
67 diseases.
68

69 **METHODS**

70 **Study Design:** Six subjects were recruited for a research study using unbiased MDS to identify
71 potential pathogens in intraocular fluid (aqueous or vitreous) (Table 1). This study was approved by

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72 the Institutional Review Board of the University of California, San Francisco (UCSF). Five of the
73 six subjects served as controls to benchmark the ability of MDS to identify a variety of pathogens;
74 Subjects 1-3 had ocular infections with herpes simplex virus 1 (HSV-1), *Cryptococcus neoformans,*
75 and *Toxoplasma gondii*, respectively. Subject 4 had non-infectious uveitis, and subject 5 had no
76 ocular inflammation but had intraocular fluid obtained at the time of a retinal membrane peel. MDS
77 was also used to investigate subject 6 who had bilateral uveitis that had defied a 16-year diagnostic
78 work-up at multiple academic centers across two continents (Table 1 and Figure 1A).
79
80 **Sequencing Library Preparation:** Samples were prepared for MDS as previously described.[6]
81 Briefly, RNA was extracted from 20-50 µL of intraocular fluid and randomly amplified to double-
82 stranded complementary DNA (cDNA) using the NuGEN Ovation v.2 kit (NuGEN, CA). cDNA
83 was tagmented with Nextera (Illumina, CA). Depletion of Abundant Sequences by Hybridization
84 (DASH), a novel molecular technique using the CRISPR (Clustered Regularly Interspaced Short
85 Palindromic Repeats)-associated nuclease Cas9 *in vitro*, selectively depleted human mitochondrial
86 cDNAs from the tagmented library, thus, enriching the MDS library for non-human (i.e., microbial)
87 sequences.[9] One sample was prepared with New England Biolabs' (NEB) Next modules to generate
88 cDNA and the NEB Next Ultra II DNA kit to convert the cDNA into sequencing libraries (NEB,
89 MA). Library size and concentration were determined using the Blue Pippin (Sage Science, MA)
90 and Kapa Universal quantitative PCR kit (Kapa Biosystems, Woburn, MA), respectively. Samples
91 were sequenced on an Illumina HiSeq 2500 instrument using 135/135 base pair (bp) paired-end
92 sequencing.[6,7]
93

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94 **Bioinformatics:** Sequencing data were analyzed using a rapid computational pipeline developed by
95 the DeRisi Laboratory to classify MDS reads and identify potential pathogens by comparison to the
96 entire National Center for Biotechnology Information (NCBI) nucleotide (nt) reference database.[6]
97 The full dataset for each subject was analyzed in less than five minutes. Briefly, paired-end reads
98 were quality filtered using PriceSeqFilter.[10] Human sequence was removed by alignment to the
99 human reference genome (hg38) using STAR.[11] Unaligned reads that were at least 95% identical
100 were compressed by cd-hit-dup (v4.6.1). These reads were then used as queries to search the NCBI
101 nt database (July 2015) using gsnapl (v2015-12-31).[12]

102

103 **RESULTS**

104 **MDS to Detect Pathogens in Uveitis**

105 MDS accurately detected viral (HSV-1), protozoan (*T. gondii*), and fungal (*C. neoformans*)
106 infections in subjects 1-3 and did not detect microbes other than known laboratory and
107 environmental contaminants in subjects 4 and 5 (Table 1).

108

109 In subject 6, MDS detected a single candidate pathogen: rubella virus (RV) in an aqueous fluid
110 specimen collected in 2014. 599 non-redundant sequence pairs mapped to both the non-structural
111 and structural open reading frames (ORFs) of the RV genome. No sequences aligning to RV were
112 present in the water control or the 18 other cerebrospinal or intraocular fluid samples sequenced on
113 the same run. No RV reads have ever been detected previously in this laboratory.

114

115 Subject 6 was a 40 year-old man with a 16-year history of inflammation in both eyes whose
116 extensive diagnostic work-up in Germany and the U.S. had been unrevealing (Table 1 and Figure

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117 1A). In 1993 he had a three-day febrile illness accompanied by a rash that spread from his back to
118 his extremities. He was diagnosed with anterior uveitis of the left eye in 1999 and in 2001,
119 developed anterior uveitis of the contralateral eye. Topical steroid and non-steroidal anti-
120 inflammatory drops were ineffective. Oral steroids were added in 2009 followed by methotrexate.
121 His inflammation did not improve after one year of combined immunotherapy, and his medications
122 were discontinued.
123
124 He presented to the Francis I. Proctor Foundation and UCSF in 2012 with moderate anterior and
125 intermediate uveitis associated with ocular hypertension and diffuse stellate keratic precipitates in
126 both eyes (Figure 1C) and asymmetrical iris atrophy leading to heterochromia (Figure 1B). These
127 findings were suggestive of viral-related uveitis, and the subject underwent an anterior chamber
128 paracentesis of the left eye. 100 μL of aqueous fluid was sent for polymerase chain reaction (PCR)
129 testing for cytomegalovirus (CMV), varicella-zoster virus (VZV), and HSV-1/2. Despite negative
130 results, suspicion for viral infection remained high. Antiviral therapy was initiated and continued for
131 three years (Figure 1A), but failed to curb the inflammation. In 2014 he had a paracentesis of the
132 right eye and a therapeutic vitrectomy of the left eye. Repeat infectious disease diagnostics were
133 unrevealing (Figure 1A).
134

135 **Confirmatory testing for RV infection**

136 A 183 nt RNA fragment was reverse transcribed and amplified from the subject's aqueous fluid
137 collected from the right eye in 2014, using a published reverse transcription PCR (RT-PCR) assay
138 for detecting the RV E1 gene.[13] Sanger sequencing confirmed that the amplicon was the RV E1
139 gene (Elim Bio, CA). This result was corroborated by the California Department of Public Health's

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140 (CDPH) Viral and Rickettsial Disease Laboratory who performed RT-PCR and Sanger sequenced
141 the 739 nt RV sequence required for genotype assignment (Sequetech Corp, CA).[14,15] RV was not
142 detected via RT-PCR in nasopharyngeal swab, urine, or tear samples collected in February 2016,
143 indicating that the subject was not actively shedding virus. Serologic testing for RV IgG was
144 positive.
145
146 An archived sample from the subject's 2014 left eye vitrectomy subsequently underwent MDS
147 using the same protocol. While the sample was not flash-frozen and was not stored to optimally
148 preserve RNA integrity, ten unique sequence pairs aligned to the RV non-structural ORF. The
149 detection of RV in both eyes corroborated the clinical suspicion of bilateral viral infection and
150 demonstrated the robustness of MDS to detect pathogens despite suboptimal sample handling.
151
152 **Characterization of RV Sequences**
153 The subject's original MDS data were combined with sequencing data obtained from four replicate
154 sequencing runs. These reads were aligned using bowtie2 v2.2.8 to the complete RV genome
155 (GenBank DQ388280.1).[16] 9,188 bp mapped, covering 95.1% of the reference genome (Figure
156 2A). This represents the most extensive coverage of an RV genome detected from any intraocular
157 sample and suggests that the RV genomes are full length.[17]
158
159 **Phylogenetic analysis of the subject's RV genome**
160 The 739 nt segment of the RV E1 gene isolated from subject 6 with MDS was compared against the
161 32 World Health Organization (WHO) RV reference strains using MUltiple Sequence Comparison
162 by Log-Expectation (MUSCLE).[18-20] His strain most closely aligned to the 1G genotype (Figure

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163 2B). Of the three lineages of the 1G genotype, the lineage containing the Stuttgart strain circulated
164 in Germany, Italy, and the United Kingdom in the early 1990s. Thus, this subject's RV strain is
165 temporally and geographically most proximate to the RV strain that was known to be circulating
166 when he developed a rash and fever in 1993 in Germany.
167
168 The RV sequence (9,188 nt) obtained from our subject includes 128 nt substitutions relative to the
169 1992 Stuttgart strain (GenBank DQ388280.1). This substitution rate of 6.97×10^{-4}
170 substitutions/site/year over the 20-year period is within two-fold of the RV evolutionary rate
171 calculated as part of epidemiologic studies evaluating person-to-person transmission (1.19×10^{-3} to
172 1.94×10^{-3} substitutions/site/year).[21] Of the 128 substitutions, 92 were synonymous (Figure 2A). Of
173 the 36 non-synonymous mutations, 19 occurred within the coding region for the E1 and E2
174 glycoproteins. Per unit length, the number of non-synonymous mutations in the E1 and E2
175 structural proteins was 4.1-fold higher than the non-structural proteins. Considering all mutations in
176 this region, the substitution rate in E1 and E2 was $1.05 \text{ x } 10^{-3}$ substitutions/site/year. We note that
177 this mutational imbalance associated with E1 and E2 compared to the non-structural proteins is
178 consistent with persistent viral replication under immunological pressure.[22]
179

180 **DISCUSSION**

181 Our results demonstrate that unbiased MDS can detect fungi, parasites, DNA viruses and RNA
182 viruses in minute volumes of intraocular fluid from patients with uveitis. In addition to correctly
183 identifying the causative agent in three infected positive control subjects (1-3) and detecting only
184 background microbial contamination in two uninfected subjects (4 and 5), MDS revealed RV in a
185 subject (6) who had a 16-year history of bilateral uveitis.

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186

187 RV is a positive sense single-stranded RNA virus in the genus *Rubivirus* of the *Togaviridae* family
188 that causes transient body rash and fever in healthy adults but can also cause devastating birth
189 defects.[23] RV has also been associated with Fuchs uveitis syndrome (FUS), a rare form of chronic
190 intraocular inflammation most often characterized by mild anterior chamber reaction, iris atrophy
191 with or without heterochromia, late onset ocular hypertension, and minimal associated visual
192 complaints.[17,24,25] In a subset of FUS patients, either RV IgG or RV RNA has been detected in
193 ocular fluid by Goldmann-Witmer coefficient analysis or RT-PCR, respectively.[17,24,26] These tests
194 are only validated for ocular fluid at a few centers in Europe and are not diagnostically available in
195 the U.S.

196

197 The protracted diagnostic challenge in our subject is three-fold: (1) diagnostic tests are lacking for
198 ocular inflammation, (2) the subject's clinical findings were not consistent with FUS until many
199 years after disease onset, and (3) the subject's relevant infectious exposure occurred six years prior
200 to the onset of his ocular symptoms. This case highlights the advantage of an hypothesis-free
201 approach in which a single MDS assay can detect a multitude of pathogens that may or may not
202 have been previously associated with a particular clinical syndrome.

203

204 The identification of RV RNA in our subject's eyes underscores current challenges in infectious
205 disease surveillance. The WHO declared RV eliminated in the U.S. in 2005 as a result of effective
206 and long-standing vaccination policies, but RV remains a threat throughout much of the world.[27,28]
207 Our subject's ocular inflammation predated his measles, mumps and rubella (MMR) vaccination by
208 seven years, and his RV strain most closely matched the strain circulating in his home country of

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209 Germany at the time of his rash and fever in 1993, and not the vaccine strain (Figure 2B). This is
210 consistent with the notion that RV likely seeded his eyes during this primary infection. Although his
211 immune system cleared the infection peripherally, RV sequestered in the ocular compartment and
212 persisted presumably due to relative immune privilege. Indeed, our analysis of the RV genome
213 provides the first molecular evidence for active RV replication in FUS. Ocular RNA virus
214 sequestration is not a phenomenon relating solely to RV, as Ebola virus was recently detected in the
215 ocular fluid of a patient nine weeks after resolution of his viremia.[4] Using RT-PCR for RV on our
216 subject's tears, we were not able to detect shedding of RV, although longitudinal studies are
217 required to determine whether intermittent shedding through tears can occur. As we devise
218 strategies to rapidly identify and control emerging and re-emerging infectious diseases, expanding
219 the scope of pathogen detection to the eyes and other immune privileged sites may be of critical
220 importance.
221
222 Diagnostic tests for intraocular infection fundamentally differ from those for systemic infections
223 because of the small sample volume that can be safely obtained from the eye. Unbiased MDS may
224 circumvent this limitation, as it detects many infectious organisms with a single assay requiring as
225 little as 20 μL of intraocular fluid. Not only does MDS have the potential to alter the paradigm for
226 infectious disease diagnostics in ophthalmology, but it may also provide another valuable public
227 health tool to surveil for re-emerging and emerging infectious diseases in immune privileged body
228 sites.
229

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230 **CONTRIBUTORS**

231 TD and MRW contributed equally and therefore are co-first authors. JLD and NRA conceived the
232 study. JLD, MRW, and TD developed study protocol and design, and were responsible for the study
233 implementation and project management. TD, MRW, LMK, Emily D. Crawford, and Eric D. Chow
234 performed library preparation and sequencing. JLD, MRW, and TD performed statistical analysis.
235 TD, MRW, and KAK performed rubella RT-PCR. DX and JKH supervised the confirmatory rubella
236 RT-PCR at the CDPH. TD, NRA, JG, and JMS obtained clinical samples and participated in patient
237 care. TD, MRW, and JLD wrote the first draft of the article. All authors contributed to the
238 interpretation of the data and the writing and editing of the article.

239

240 **DECLARATION OF INTEREST**

241 We declare no competing interest.

242

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339 **FIGURE LEGENDS**

340

341 **Table 1: Results of Unbiased Metagenomic Deep Sequencing (MDS) and Conventional**
342 **Diagnostic Tests on Intraocular Fluid Samples.** MDS correctly identifies known infections in
343 subjects 1-3. Subjects 4 and 5 had non-infectious ocular disease and had negative MDS testing for
344 pathogens, defined as the presence of no microbial sequences other than known laboratory and
345 environmental contaminants. Rubella virus was identified via MDS in subject 6 and confirmed by
346 the California Department of Public Heath's RT-PCR assay. Abbreviations: Pos, positive; Neg,
347 negative; NA, not applicable; RT-PCR, reverse transcription polymerase chain reaction; HSV-1,
348 herpes simplex virus-1; HSV-2, herpes simplex virus-2; VZV, varicella zoster virus; CMV,
349 cytomegalovirus; *T. gondii, Toxoplasma gondii*; RV, rubella virus; *C. neoformans, Cryptococcus*
350 *neoformans*; RE, right eye; LE, left eye.

351

352 **Figure 1: Clinical Course and Ocular Findings of a 40 Year-Old Man With Bilateral,**
353 **Idiopathic Chronic Anterior and Intermediate Uveitis.** Panel A shows Subject 6's clinical course
354 spanning 22 years. Panel B shows different colored irises (heterochromia) between the right and left
355 eyes (top panels) and transillumination defects that are prominent in the left eye because of iris
356 atrophy (lower panels). Panel C shows diffused aggregates of inflammatory cells (keratic
357 precipitates; red arrows) on the endothelium of the cornea. Abbreviations: HSV, herpes simplex
358 virus; VZV varicella zoster virus; CMV, cytomegalovirus; PCR, polymerase chain reaction; RE,
359 right eye; LE, left eye; MMR, measles/mumps/rubella vaccine; MTX, methotrexate.

360

361 **Figure 2: Identification of Rubella Virus (RV) by Metagenomic Deep Sequencing.** Panel A

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362 illustrates how the 9,188 nucleotide (nt) paired-end sequence reads obtained from sequencing the
363 RNA extracted from the subject's aqueous fluid aligned to the most closely matched RV genome
364 (DQ388280.1). 95.1% of the total RV genome is represented. Positions of synonymous (black
365 vertical lines) and non-synonymous (red vertical lines) variants are shown. Of the 128 substitutions,
366 92 were synonymous, and 36 were non-synonymous. Of the 36 non-synonymous mutations, 19
367 occurred within the coding region for the E1 and E2 glycoproteins. Per unit length, the number of
368 non-synonymous mutations in the E1 and E2 proteins was 4.1-fold higher than the non-structural
369 proteins. The cyan marker above the E1 gene represents the 739 nt sequence window recommended
370 by the World Health Organization (WHO) for RV genotyping. Panel B is a phylogenetic analysis of
371 the subject's RV strain obtained from MDS with 32 WHO reference strains, GUZ_GER92
372 (Stuttgart strain), and the RV27/3 vaccine strain, demonstrating that the subject's RV sequence was
373 most closely related to the genotype 1G viruses and not the vaccine strain.
374
375

Table 1

Subject	Clinical Diagnosis	Sample Type	MDS	PCR					RT- PCR	Culture
				HSV-1	HSV-2	VZV	CMV	T. gondii	RV	
1	Anterior uveitis	Aqueous fluid	***HSV-1 (441 reads)***	**Pos**	Neg	Neg	Neg	Neg	NA	NA
2	Panuveitis	Vitreous fluid	***C. neoformans (9,117 reads)***	Neg	Neg	Neg	Neg	Neg	NA	***C. neoformans***
3	Panuveitis	Vitreous fluid	***T. gondii (2,638 reads)***	Neg	Neg	Neg	Neg	**Pos**	NA	NA
4	Panuveitis (steroid responsive)	Aqueous fluid	Neg	Neg	Neg	Neg	Neg	Neg	NA	Neg
5	Epiretinal membrane (non-inflammatory)	Aqueous fluid	Neg	NA	NA	NA	NA	NA	NA	NA
		Vitreous fluid	Neg							
6	Anterior & intermediate uveitis	Aqueous fluid, right eye	**RV (599 reads)**	NA	NA	NA	NA	NA	**Pos**	NA
		Vitreous fluid, left eye	***RV (10 reads)***	Neg	Neg	Neg	Neg	Neg	NA	NA
	Control	H_20	Neg	NA	NA	NA	NA	NA	NA	NA

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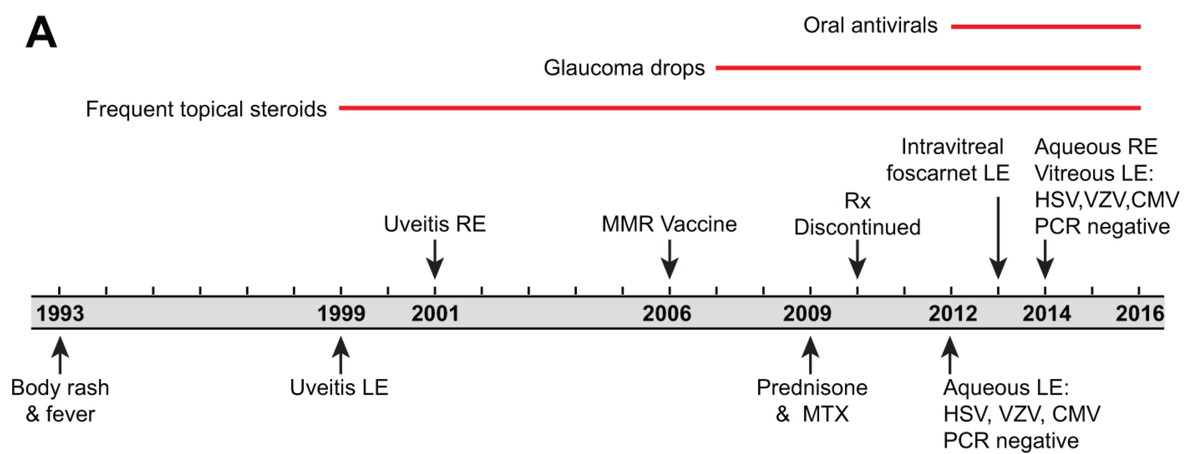

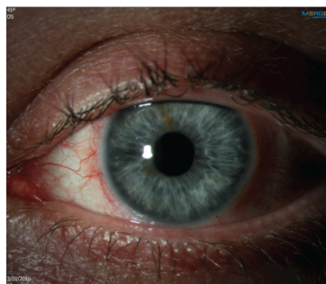

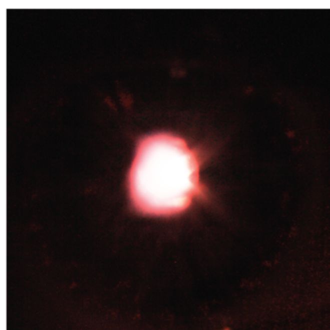

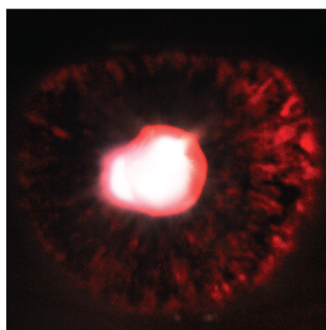

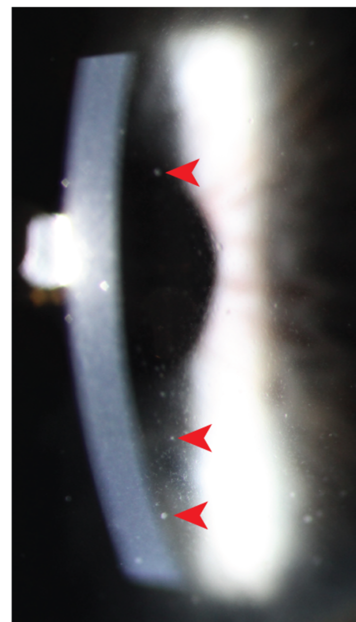

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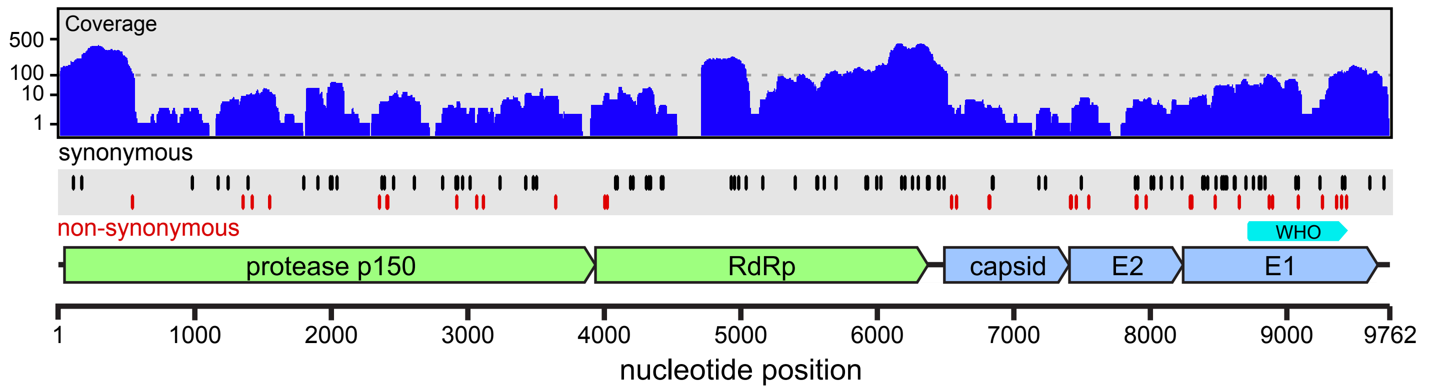

B

RVi/Beijing.CHN/79_2A
RVi/Beijing.CHN/80_2A
RVi/TelAviv.ISR/68_2B
RVi/Anqing.Anhui.CHN/00_2B
RVi/Seattle.USA/16.00_2B
RVi/Moscow.RUS/67/_2C
RVi/Moscow.RUS/97_2C
RVi/NJ.USA/61_1a
RVi/BEL/63_1a
RVi/Toyama.JPN/67_1a
RVi/PA.USA/64_1a
RA27/3_Vaccine
RVi/Dangshan.Anhui.CHN/00_1F
RVi/Linqing.Shandong.CHN/00_1F
RVi/Jerusalem.ISR/75_1B
RVi/Bene-Berak.ISR/79_1B
RVi/Tiberius.ISR/88_1B
RVi/Tomsk.RUS/05_1h
RVi/Minsk.BLR/28.05/2_1h
RVi/Minsk.BLR/29.04/1_1G
RVi/Ontario.CAN/05_1G
UCSF Uveitis Patient Sample
GUZ_GER92 (Stuttgart Germany)
RVi/UGA/20.01_1G
RVi/Milan.ITA/46.92_1i
RVi/Pavia.ITA/21.91_1i
RVi/PAN/99_1C
RVi/SLV/02_1C
RVi/LA.CA.USA/91_1C
RVi/Tochigi.JPN/04-1_1j
RVs/Miami.FL.USA/32.02_1j
RVi/Saitama.JPN/94_1D
RVi/Tokio.JPN/90_1D
RVi/Dezhou.Shandong.CHN/02_1E
RVi/MYS/01_1E