

1 **Effectiveness of real-time PCR for diagnosis and**
2 **prognosis of varicella-zoster virus keratitis**

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10 Running title: Real-time PCR for VZV keratitis

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21 **Abstract**

22 **Purpose:** To determine the efficacy of real-time PCR for the diagnosis and prognosis
23 of varicella-zoster virus (VZV) keratitis.

24 **Study design:** Retrospective case series.

25 **Methods:** Patients: 545 consecutive patients with keratitis were examined to quantify
26 copy numbers of VZV DNA by real-time PCR. Association of copy numbers of DNA of
27 VZV to clinical signs and disease course was assessed using logistic regression
28 analysis and Cox proportional hazard model.

29 **Results:** Of the 545 eyes, 38 eyes (6.9%) were diagnosed as VZV keratitis. The copy
30 numbers of the DNA of VZV (median: $10^{4.19}$ copy) was significantly associated with
31 diagnosis of VZV keratitis with the highest odds ratio (OR) of 3390 (for median copy)
32 compared to clinical signs. Diagnostic accuracy of the VZV DNA copy indicated good
33 diagnostic value of area under the curve (0.92) by receiver operating characteristic
34 analysis, and detection of unrelated VZV DNA from the cornea was very rare (0.2%).
35 When the VZV DNA copy and clinical signs were assessed for association with the
36 disease course after herpes zoster ophthalmicus, the disease duration was
37 significantly prolonged in VZV keratitis cases with higher numbers of VZV DNA copies,
38 iritis, and history of recurrences. The amount of VZV DNA led to a continuous risk to
39 prolong disease duration until the ocular inflammation subsides (hazard ratio (HR)

40 0.17, 95%CI: 0.07 - 0.42, for median copies).

41 **Conclusions:** Higher VZV DNA copy numbers are associated with the refractoriness
42 of VZV keratitis, and its evaluation may be a clinically useful way to diagnose and
43 manage VZV keratitis.

44

45

46 **Keywords:**

47

48 varicella-zoster virus keratitis, real-time PCR, herpes zoster

49 ophthalmicus

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52 **Introduction**

53 Herpes zoster contributes significantly to morbidity in elderly individuals and is mainly
54 caused by a reactivation of the varicella-zoster virus (VZV). Almost one-third of
55 individuals are estimated to be affected by herpes zoster, [1, 2] and up to 20% of
56 herpes zoster infections are expressed as herpes zoster ophthalmicus (HZO).[2, 3]
57 Patients with HZO have significantly higher risks of strokes and post-herpetic
58 neuralgia. [4-6] Importantly, an ocular complication is observed in 35.1% to 65% of
59 HZO patients.[7, 8]

60

61 However, the etiology of the ocular complication has not been determined. For
62 example, the VZV DNA in the ocular lesions of VZV keratitis is occasionally not
63 detected by conventional PCR. [9] For VZV keratitis cases positive for VZV DNA after
64 HZO, VZV DNA was believed to disappear soon after the onset. Thus, it is still unclear
65 whether active keratitis lesions are caused by VZV replication or an inflammatory
66 response of the host.

67

68 The purpose of this study was to determine the relationship between the presence of
69 VZV DNA in the eye and the diagnosis of HZO. To accomplish this, we quantified the

70 VZV DNA by quantitative real-time PCR (qPCR) and assessed the association of the
71 copy numbers with the clinical signs with and without previous HZO. We then
72 analyzed course of ocular complications after HZO. We shall show that VZV DNA was
73 a significant risk factor for prolonged ocular complications after HZO.

74

75

76 **Materials and Methods**

77 ***Patients eligibility and diagnostic criteria of VZV keratitis***

78 The medical records of 545 consecutive cases with clinically diagnosed keratitis were
79 reviewed, and all were examined at the Tottori University Medical Hospital between
80 November 2005 and September 2016. All of these cases had undergone qPCR for
81 VZV. Of these 545 cases, 283 patients were men, and the mean age was 56.1 ± 23.1
82 years.

83

84 The diagnosis of HZO was based on the presence of primary skin rashes with
85 erythema within the ophthalmic dermatome as described in detail.[9] Acute VZV
86 keratitis was diagnosed when keratitis was present with preceding skin rashes <90
87 days from the onset of the skin lesions.[2] In cases without preceding skin rash with
88 positive VZV by conventional PCR, a diagnosis of acute VZV keratitis was made by

89 the responsiveness to oral valaciclovir/acyclovir or topical acyclovir ointment, or the
90 combination of these anti-herpetic drugs and steroid treatments. Chronic VZV
91 keratitis was defined to be present when the activity required anti-viral drugs or
92 steroids for ≥ 90 days from the onset of skin lesions.[2] Recurrent disease was defined
93 to be present, when keratitis recurred ≥ 90 days after the resolution of the signs
94 without the use of anti-viral drugs or steroids. DNA samples were collected at each
95 outpatient visit until the clinical symptoms were resolved for the VZV keratitis cases.

96

97 A diagnosis of herpetic keratitis was made when herpes simplex virus (HSV) was
98 detected by PCR. A diagnosis of other non-VZV keratitis, including bacterial keratitis,
99 fungal keratitis, acanthamoeba keratitis, adenoviral keratitis, or autoimmune keratitis,
100 was made by conventional culturing, smearing, and PCR as described.[10-13]

101

102 This study was approved by the Institutional Review Board of Tottori University,
103 Faculty of Medicine, Tottori, Japan. An informed consent was obtained prior to the
104 procedures from all of the participants after an explanation of the procedures to be
105 used.

106

107 **Quantitative real-time PCR**

108 Samples were collected from the ocular surface and cornea by rinsing them with 400
109 μ l of saline without touching the eyelids and skin lesions. DNA was extracted from the
110 samples with the QIAamp DNA mini kit (Qiagen, Hilden, Germany) and were
111 amplified with the LightCycler (Roche, Basel, Switzerland) using QuantiTect Probe
112 PCR kit (Qiagen) and primers (Supplementary Table 1).[14, 15] To determine the
113 copy numbers of the DNA of VZV, a standard curve was generated with serial
114 dilutions of synthesized DNA fragments of the VZV polymerase gene.[14] The limit of
115 detection at a 95% detection probability was 49 copies/assay. VZV copy number was
116 adjusted by measurement of human GAPDH copy. [15]

117

118 **Statistical analyses**

119 Data are presented as the means \pm standard deviations (SDs). For bilateral cases,
120 the more severely affected eye was used for the statistical analyses. Cox proportional
121 hazard model with shared frailty was used to calculate the hazard ratio (HR) during
122 the course of the disease. Statistical analyses were conducted using Stata 14. A *P*
123 <0.05 was considered significant.

124

125 **Results**

126 **Diagnostic efficacy of qPCR for VZV keratitis**

127 Patients with corneal ulcer or inflammatory keratitis which was suspected to be
128 caused by VZV infection or required the exclusion of VZV for diagnosis were studied.
129 Of the 545 eyes, 38 eyes (6.9%) were diagnosed with VZV keratitis. Thirty-seven
130 eyes had HZO keratitis and 1 eye had varicella keratitis. The mean age of the HZO
131 keratitis patients was 63.2 ± 20.0 years (Table 1).
132
133 qPCR for VZV was positive for 32 eyes (5.9%) of all the cases, and 30 eyes of PCR
134 positive cases had HZO keratitis and 1 eye had varicella keratitis. All the VZV keratitis
135 cases had prior periocular skin rashes, and 35 eyes (92.1%) were within 3 months of
136 the initial visit. The percentage of eyes with ocular shedding of VZV DNA by non-VZV
137 infection was very low in the diseased corneas (0.2%).
138
139 For the eyes diagnosed with VZV keratitis, the mean copy number was $10^{4.21} \pm 10^{2.61}$,
140 and the median copy number was $10^{4.19}$. VZV DNA was significantly associated with
141 VZV keratitis (Odds ratio (OR): 3390 for $10^{4.19}$ (median) copies, 6.9/log copy, after
142 age and GAPDH adjustments, $P = 0.000$; Table 2). Periocular skin rashes
143 (irrespective of dermatomal distribution and within the prior 3 months) also
144 significantly associated with VZV keratitis, followed by ocular hypertension, iritis with
145 mutton fat keratic precipitates, dendritic lesions, and scleritis (Table 2).

146

147 Next, we determined how these clinical signs and VZV DNA copy numbers are
148 related to the diagnosis of VZV keratitis (Table 2). VZV DNA qPCR had a sensitivity of
149 81.6% and specificity of 99.8%. The likelihood ratio, which denotes the efficiency of
150 the test, was the highest at 408 (Table 2). In contrast to the clinical signs, qPCR for
151 VZV had higher positive predictive value and likelihood ratio, indicating that qPCR is
152 accurate for both the negative and positive results for the diagnosis of VZV keratitis.

153

154 Of the 545 eyes, periocular skin rashes were observed in 49 eyes (9.0%), and 15 of
155 these eyes (30.6%) were not related to VZV infection. The sensitivity and specificity of
156 the skin rashes was 92.1% and 97.2%, and its likelihood ratio was lower at 32.9.

157

158 Other clinical signs, including ocular hypertension, iritis, corneal dendritic lesion, and
159 scleritis, were also significantly associated with VZV keratitis, however, lower in
160 positive predictive value and likelihood ratio.

161

162 Next, we assessed the diagnostic accuracy of these signs in comparison to that of
163 qPCR using receiver operating characteristic analysis (ROC; Figure 1). The area
164 under the curve (AUC) for periocular skin rashes calculated as reference was 0.95
165 (95%CI: 0.90 – 0.99). The AUC for VZV qPCR was 0.92 (95%CI: 0.86 – 0.98) after

166 GAPDH adjustments and was not different for the skin rashes. The AUC of dendritic
167 lesions, ocular hypertension, and iritis was 0.66 (95%CI: 0.58 – 0.74), 0.62 (95%CI:
168 0.55 -0.70), and 0.60 (95%CI: 0.53 – 0.67) respectively, and their diagnostic accuracy
169 was significantly lower than that of qPCR ($P=0.000$).

170

171 **Association of copy numbers of DNA of VZV to clinical signs and disease**

172 **course**

173 We determined whether the copy numbers of the DNA of VZV was significantly
174 associated with the clinical signs or outcomes using logistic regression analysis
175 (Table 3). As expected, the copy number of DNA at the first visit was significantly
176 associated with the presence of periocular skin rashes (OR: 100.6 for median copies,
177 95% CI: 22.9-441.9, $P=0.000$, age and GAPDH adjusted). Notably, the copy number
178 of DNA was significantly associated with iritis with OR of 6.0 indicating that high VZV
179 copy number is especially associated with intraocular inflammation. This was
180 followed by ocular hypertension (OR: 3.7) and dendritic lesion (OR: 3.5).

181

182

183 Therefore, we determined whether the amount of the DNA of VZV at the initial
184 diagnosis of keratitis can predict the refractoriness and prognosis of HZO keratitis. A

185 higher copy number of VZV DNA at the first visit was significantly correlated with the
186 duration of the disease ($\rho = 0.53$, $P = 0.0007$, Spearman correlation analysis). In
187 refractory cases with iritis as the intraocular inflammation, the VZV genome was
188 detected until all clinical signs of the keratitis were not detected (Figure 2).

189

190 When the history of recurrences was evaluated in HZO keratitis patients, 8 eyes
191 (21.6%) had a history of recurrences. Of these 8 eyes, 2 eyes (25%) were from
192 immune compromised patients. Immune compromised patients had significantly
193 higher number of recurrences by a 50.6% increase of chance ($P = 0.019$ after VZV
194 DNA copy adjustment).

195

196 The mean duration of the HZO keratitis was 119 days (95%CI: 82 – 155). Kaplan
197 Meier survival analyses were performed to determine whether high copy numbers
198 (more than the median) at the first visit, the clinical characteristics and previous
199 recurrences were associated with the disease duration. The results showed that high
200 VZV copy numbers (\geq median; $P = 0.008$, log-rank test), iritis ($P = 0.01$), and history of
201 recurrences were significantly associated with the duration of the disease ($P = 0.006$,
202 Figure 3). Importantly, iritis with high VZV copy number had the most significant effect
203 on the disease duration.

204

205 During the course of the disease process, we monitored the copy numbers of the
206 DNA of VZV until HZO keratitis resolved. DNA of VZV was continuously detected at
207 the outpatient visits with declining tendency, and become detectable when signs
208 become absent. Then, we calculated the hazard ratio of VZV copy numbers during
209 the course (including the first visit) on the disease duration. VZV copy numbers
210 indicated highly significant HR, and was 0.17 for median of VZV copies (95% CI:
211 0.07- 0.42, $P=0.000$). Thus, detection of VZV copy number predicted a prolonged
212 disease course, and the disease prolonging effect declines with its decrease and
213 become negligible when it is not detected. HR indicated that the keratitis was six
214 times more likely not to be resolved on a given date when the VZV copy numbers
215 exceeded median. Presence of iritis were also significant risk factor associated with
216 longer disease duration with comparable HR (HR, 0.14, 95% CI: 0.04- 0.49, $P =$
217 0.002). When the history of recurrences was assessed, the hazard ratio was
218 calculated to be 0.15 (95%CI: 0.03 – 0.69, $P = 0.01$), indicating that recurrences were
219 also associated significantly with a prolongation of the disease duration.

220

221

222

223 **Discussion**

224 Our results showed that qPCR is highly efficacious for diagnosing ocular VZV
225 infection. Using qPCR, we here show two important findings. First, in case of high
226 viral loads at ocular surface after HZO, the prolongation of keratitis was significantly
227 associated with continuous viral production. Second, VZV keratitis was strictly
228 associated with previous HZO, although VZV iritis without keratitis can often occur
229 without noticeable history of HZO. These information is important for the
230 management of refractory ocular complications after HZO because ocular
231 inflammation was previously thought to be an anti-viral immune response without viral
232 production and often treated without antiviral medications.

233

234 Zaal et al examined the inflammation of VZV keratitis after HZO by conventional
235 PCR.[9] Because the inflammation often persisted after the VZV genome became
236 undetectable, they suggested that inflammation was an important component of the
237 pathology of VZV keratitis.

238

239 Considering the inflammatory aspect of ocular complications after HZO, a
240 combination of antiviral drugs and steroids is generally used, however consensus has
241 not been reached on how antiviral agents should be used. [16] The general belief was

242 that the period of VZV replication in the lesion is not prolonged beyond the acute
243 phase of skin lesion stage. Currently, one-third of corneal specialists use antiviral
244 drugs for 2 weeks and 18% do not use antivirals for HZO.[16]

245

246 In chronic and refractory cases, high amounts of VZV DNA were detected for a long
247 time. If antivirals are discontinued after presumed clinical remission with significant
248 viral replication, a recurrence in the form of delayed dendritic ulcer or iritis develops
249 as ocular complications can be expected. Thus, VZV qPCR will be beneficial for
250 clinicians to optimize drug dosage and duration during the disease course when the
251 signs are resolving.

252

253 The presence of iritis was recently shown to be significantly associated with a risk of
254 recurrences or chronicity in VZV keratitis after HZO.[2] We found that iritis with low
255 copy numbers of the DNA of VZV was not a significant risk factor for refractoriness
256 (Figure 2a). Instead, a combination of high amounts of VZV DNA copy and iritis were
257 the most significant factors in the refractory cases. In the refractory or chronic cases,
258 high VZV DNA copy numbers were maintained until the inflammation subsided. This
259 suggests that prolonged viral replication and not the presence of iritis, determines the
260 disease course in refractory cases.

261

262 The results of two studies have suggested that the detection of VZV may be caused
263 by shedding and was independent of the refractoriness.[8, 17] In addition, VZV may
264 be often reactivated in the saliva of healthy individuals when under extreme
265 stress.[17] In bone marrow transplant patients, 19% have subclinical viremia without
266 signs of HZO.[3, 18] This suggested detection of viral genome was frequent, and may
267 not reflect diseases. In Japanese population, seropositivity reaches almost 100% in
268 elderly subjects. [19] However, we found that the rate of unrelated VZV shedding
269 from the eye was very low (0.2%) even in eyes stressed by non-VZV keratitis. This is
270 in marked contrast to ocular HSV infection, in which spontaneous HSV shedding in
271 tear is observed in one third of healthy subjects. [20]

272

273 Recurrences of ocular complication after HZO are frequent. The percentage of eyes
274 with recurrences range between 5 to 25% depending on the duration of the
275 observation period.[2, 21, 22] Consistent with previous reports, the recurrence
276 percentage in our study was 21.6%. Tran et al reported that a recurrence of keratitis
277 after HZO was associated with uveitis and ocular hypertension.[2] However, we could
278 not confirm any significant associations of the recurrences with these clinical signs or
279 the VZV DNA copy numbers. Instead, we found that the disease duration of single

280 episode was the only significant risk factor for recurrences. We suggest that the
281 clinical signs are secondary to the long disease course which would directly explain
282 the recurrences.

283

284 There are several limitations in our study. Our data were obtained at a tertiary referral
285 institution, and selection or referral bias may have affected our outcomes. However,
286 our data are based on 12 consecutive years of observation and should provide
287 epidemiological evidence for Asians. In addition, the outcomes from a large series of
288 VZV qPCR data from the eye have not been available.

289

290 In conclusion, VZV qPCR revealed an unexpectedly longer viral replication period
291 and provided an effective measure to assess the viral load accurately during the
292 course of the disease process. We propose that the management strategy of ocular
293 complication after HZO would be significantly improved in the future with the use of
294 VZV qPCR.

295

296

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367

Table 1 Demographic information of patients and VZV keratitis

Total 545 eyes	HZO keratitis N=37	Varicella keratitis N=1	Non-VZV-associated keratitis N=507
Age	63.2 ± 20.0	2	55.7 ± 23.1
Male	24 eyes (64.9%)	0 eyes	259 eyes (51.1%)
Asthma	3 eyes (8.1%)	0 eyes	11 eyes (2.2%)
Eczema	1 eyes (2.7%)	1 eyes	31 eyes (6.1%)

368

369

Table 2 Association of VZV DNA copy numbers and clinical signs of VZV keratitis and evaluation of their diagnostic accuracy

Total 545 eyes	ratio of positive eyes	Odds ratio after age adjustment	positive predictive value	negative predictive value	Sensitivity	Specificity	likelihood ratio
		3390* (135-85383)					
VZV DNA copy	32* (5.9%)	:median copy 6.9* (3.2-15.0)	96.9% (80.3-99.6%)	98.6% (97.2-99.3%)	81.6% (65.8-91.1%)	99.8% (98.6-100%)	408 (47-3037)
		:log copy					
Periocular skin rashes	49* (9.0%)	701* (162 - 3057)	71.4% (57.2-82.4%)	99.4% (98.1-99.8%)	92.1% (77.9-97.5%)	97.2% (95.3-98.3%)	32.9 (16.6-57.4)
Ocular hypertension	47* (8.6%)	5.8* (2.7 – 12.6)	25.5% (15.0-40.0%)	94.8% (92.4-96.4%)	31.6% (18.7-48.0%)	93.1% (90.5-95.0%)	4.6 (2.0-9.6)
Iritis with mutton fat keratic precipitates	39* (7.2%)	5.4* (2.4 - 12.5)	25.6% (14.3-41.7%)	94.5% (92.1-96.2%)	26.3 (14.6-42.6%)	94.3% (91.9-96.0%)	4.6 (1.8-10.7)
Corneal dendritic lesion	85* (15.6%)	5.0* (2.5 - 10.1)	20.0% (12.8-30.0%)	95.4% (93.1-97.0%)	44.7% (29.7-60.8%)	86.6% (83.3%-89.3%)	3.3 (1.8-5.7)
Scleritis	28** (5.1%)	3.9*** (1.5 – 10.4)	21.4% (9.8-40.7%)	93.8% (91.4-95.6%)	15.8% (7.2-31.3%)	95.7% (93.5-97.1%)	3.7 (1.1-10.8)

*: statistically significant, *: P=0.000, **: P=0.009, ***: P=0.006, 95% confidence interval (95%CI)

373

Table 3 Association of VZV copy numbers to clinical signs and characteristics

	Odds ratio (Median copy)	95% confidence interval	<i>P</i> value
Periocular skin rashes	100.6	22.9 – 441.9	0.000
Iritis (with mutton fat keratic precipitates)	6.0	2.4 – 15.2	0.000
Ocular hypertension	3.7	1.6 - 8.9	0.003
Corneal dendritic lesion	3.5	1.6 – 7.9	0.002

Logistic regression analysis after age and GAPDH adjustment

374

375

376

377 **Figure captions**

378 **Figure 1.** Diagnostic accuracy of qPCR for VZV and clinical signs.

379 The sensitivity and specificity of VZV qPCR and clinical signs to diagnose VZV
380 keratitis are plotted to determine the area under the curve (AUC) as diagnostic
381 accuracy by receiver operating characteristic analysis. The VZV DNA copy number
382 has a very high accuracy comparable to that for skin rashes calculated as reference
383 and is significantly better than the other signs.

384

385 **Figure 2.** Case of VZV keratitis with duration of VZV disease. A 53-year-old man
386 presented with herpes zoster ophthalmicus (HZO) with periocular skin rashes. One
387 month later, he developed VZV keratitis with corneal edema and iritis, and the copy
388 number of the DNA of VZV was 4.7×10^5 . The elevated copy number was present for
389 4 months.

390 **a** VZV keratitis with stromal infiltration (arrow) and iritis with VZV copy of 4.5×10^5 at 2
391 months after the onset of HZO.

392 **b** Prolonged elevation of VZV DNA copy number during the course of the disease.

393

394 **Figure 3.** Association of duration of VZV keratitis to VZV DNA copy number and

395 clinical characteristics.

396 Disease duration was analyzed using Kaplan Meier survival analysis and plotted on

397 non-healing rate. **a** High VZV DNA copy number (\geq median ($10^{4.19}$ copies); $P = 0.008$,

398 log-rank test), the presence of iritis ($P = 0.01$), and **b** history of recurrences ($P =$

399 0.006) were significantly associated with the disease duration.

400

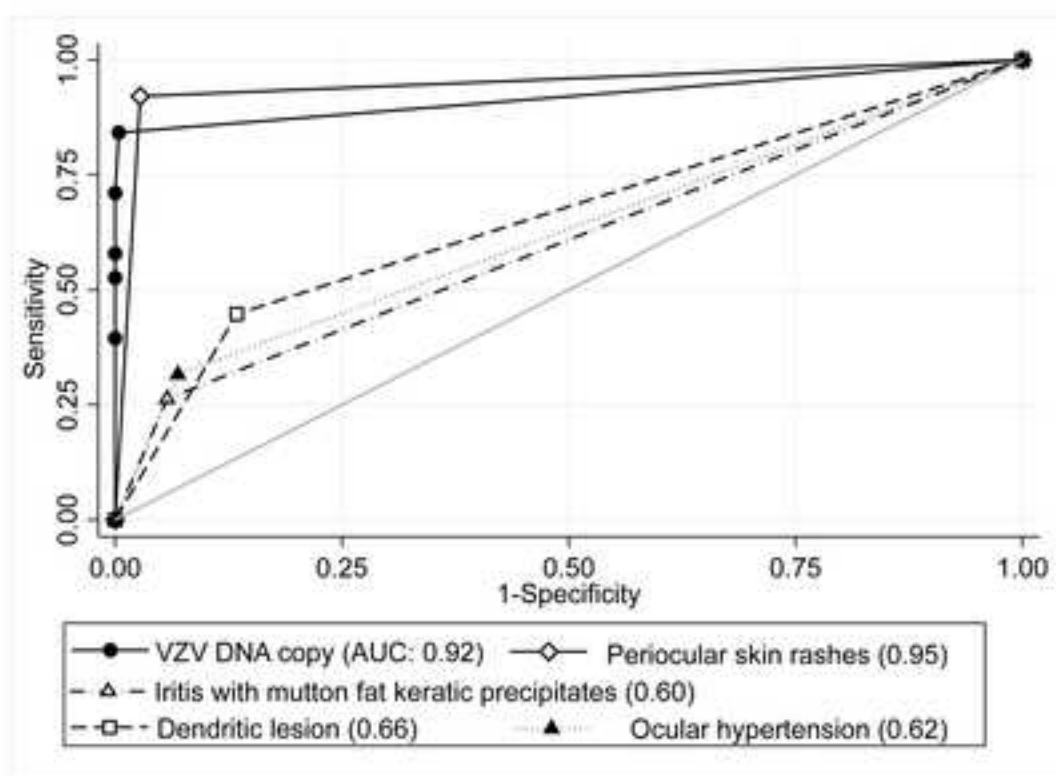


Figure 1

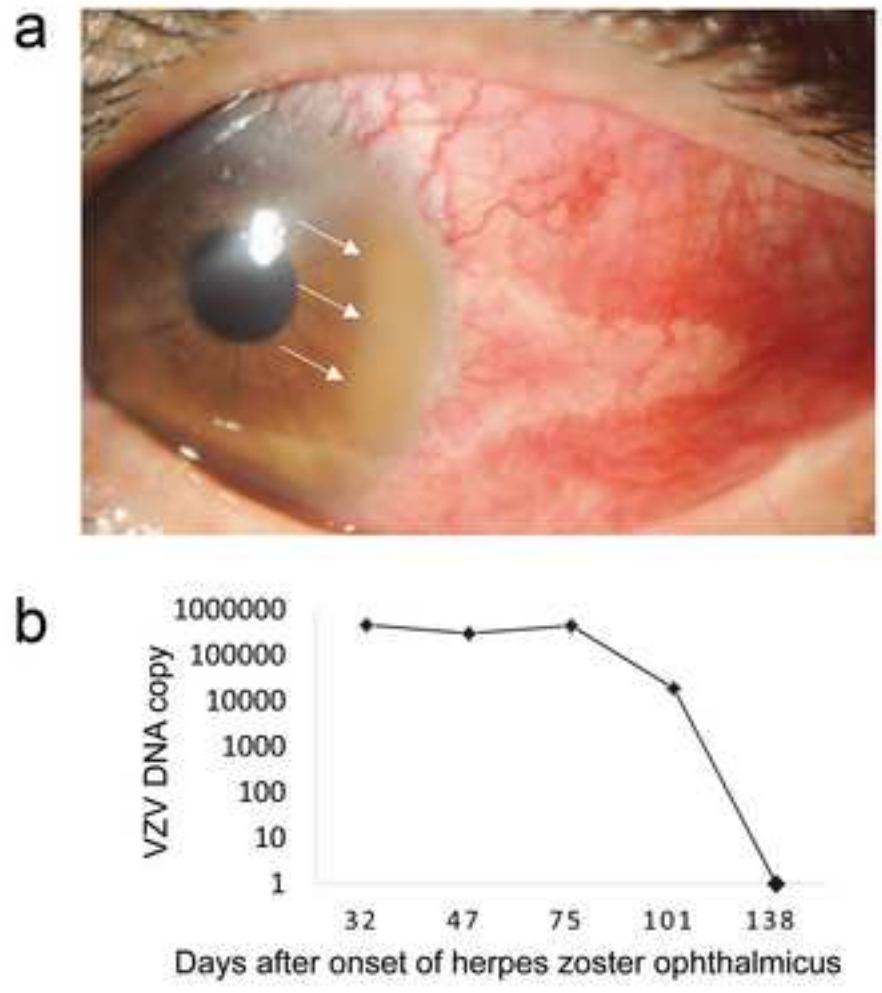


Figure 2

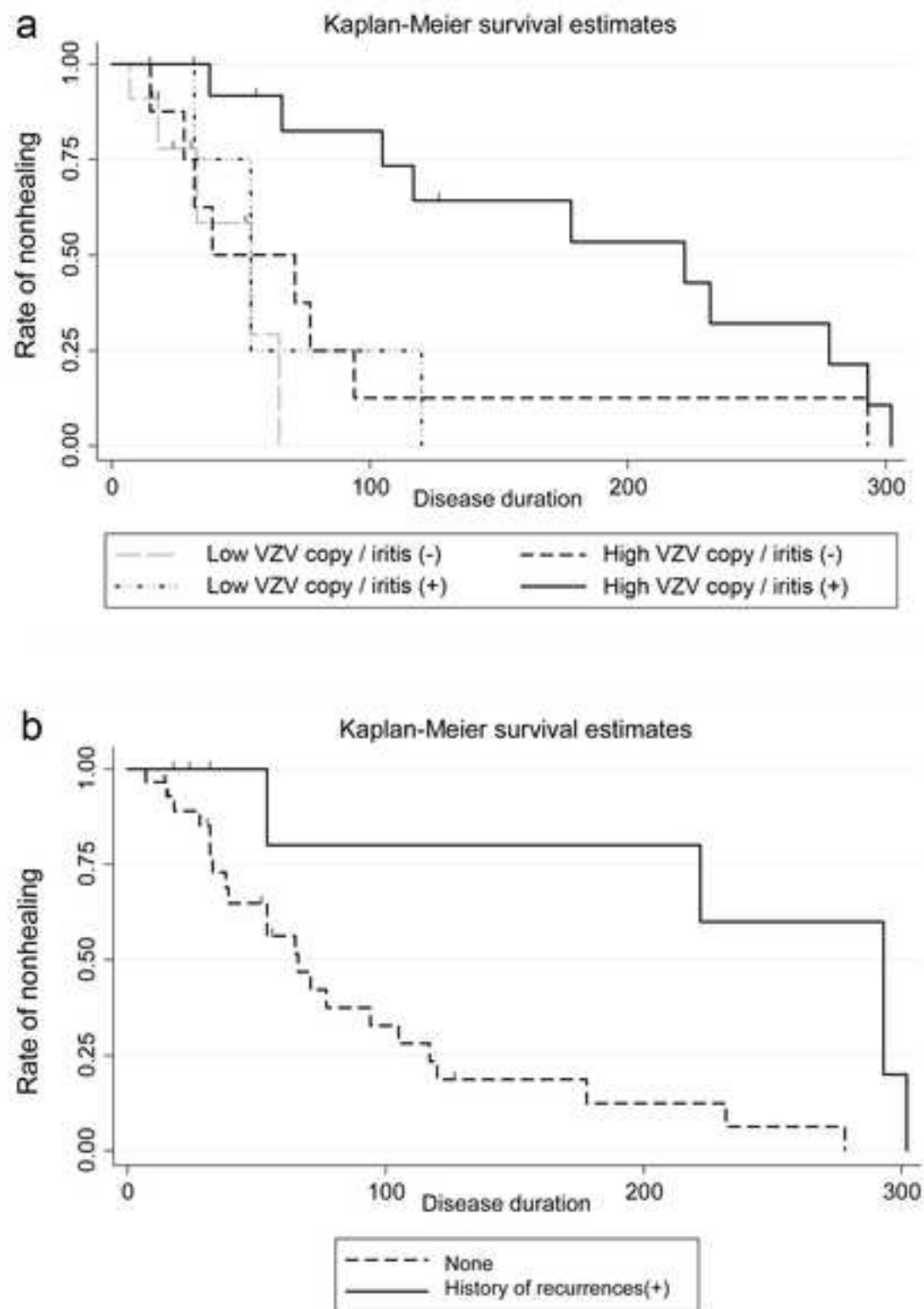


Figure 3