

Ecosphere

Appendix S1

**Impacts of sequential and simultaneous coinfection by major amphibian pathogens
on disease outcomes**

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Evaluating *Hymenochirus boettgeri* susceptibility to Ranavirus FV3

Methods

In a series of pilot experiments, we exposed *H. boettgeri* adults to various doses of Rv (Ranavirus FV3) using three inoculation methods: water bath exposure, oral administration, and intraperitoneal injection. We assigned four frogs to each dose in each inoculation method for a total of 40 animals (20 of each sex). Pilot frogs were the same age (5 months old) and had an approximate starting size (SVL = 27.15 ± 2.05 mm) and mass (1.27 ± 0.21 g). Before inoculation, a random subset of 40% was confirmed negative for Rv by skin and buccal swabs.

Water bath exposure involved placing animals into 3 mL of autoclaved pre-conditioned RO water with the desired inoculum dose (10^6 or 10^7 PFU/mL, where PFU = plaque forming units) in a 50 mL falcon tube for 3 hours. Oral administration involved injecting 40 μ L of liquid inoculum into the mouth three times, spaced 30-90 minutes apart to minimize animal stress and regurgitation of the total 120 μ L of liquid containing the desired dose (10^6 or 10^7 PFU total). Intraperitoneal injection involved gently inserting a 30-gauge needle into the peritoneal cavity, just anterior to and above the legs, slowly injecting 30 μ L of liquid containing the desired inoculum dose (10^4 , 10^5 or 10^6 PFU total). For each inoculation method, sham exposures used the same volumes of APBS. APBS was also used for all dilutions.

Skin and buccal swabs were performed on day 0, 4, 14, and time of death or experimental endpoint (day 42). Skin swabs mirrored a standard Bd detection protocol (Hyatt et al., 2007), and buccal swabs were performed by turning a sterile swab head 180° in both directions just inside the mouth, five times. Protocols for DNA extraction and qPCR of skin and buccal swabs are described in Methods: *Bd and Rv Quantification from Swabs*.

Within approximately 10 minutes following euthanasia, a small left-side portion of the liver and both kidneys were removed using bleach-sterilized dissection scissors and forceps. The mass of all tissue samples was recorded, ensuring none exceeded 30 mg (recommended for DNA extraction protocol). Tissues were stored in 96% ethanol at -80°C and washed twice with PBS prior to DNA extraction using the Qiagen Blood and Tissue DNeasy extraction kit. DNA extraction followed manufacturer guidelines with the following changes: 1.5 hours of lysis time, with vortexing every 20 minutes, an added membrane-drying step, and twice eluting 50 µL of UV-sterilized nuclease-free water for increased yield recovery. qPCR for viral quantification in tissues mirrored the protocol described in Methods: *Bd and Rv Quantification from Swabs*. However, dilution of tissue DNA extractions was sometimes necessary to eliminate matrix effects on qPCR chemistry. Additionally, qPCR quantities were not only multiplied by 40 (dilution factor used to reflect total extracted MCP gene copies), but were also scaled to the mass of the tissue extracted. Final viral loads were reported as MCP gene copies per 10 mg of extracted kidney tissue, or per 50 mg of extracted liver tissue, in order to reflect total viruses contained in the approximate typical mass of each whole organ.

Kaplan-Meier survival analysis was performed in the same manner as described in Methods: *Statistical Analysis*. To evaluate variations in transmission potential between treatments at specific time points, we performed a Kruskal-Wallis rank sum test using *kruskal.test* on qPCR-derived values because the data were not normally distributed (McKight and Najab, 2010), and we performed post-hoc analysis using the Dunn test with Bonferonni correction using *dunn.test* (Alexis Dinno, 2014).

Results

Survival curves depicted in Figure S1 demonstrate that *H. boettgeri* is susceptible to Ranavirus FV3 infection and exhibits variable survival outcomes depending on the dose and inoculation method. However, pairwise log-rank tests did not reveal any statistically significant differences in survival between dose exposures ($p > 0.05$), possibly due to low power. Water bath exposure was chosen for the coinfection experiment for the following reasons: this exposure was the least invasive inoculation method, was likely to best simulate natural infection, displayed a preliminarily dose-dependent trend in mortality, produced an expected viral load increase through time as detected by swabs (Figure S2), and allowed us to simultaneously inoculate frogs with the aquatic fungus Bd for coinfections.

Another goal of the pilot was to assess the efficacy of different viral load detection methods: skin swabs, buccal swabs, liver samples, and kidney samples. Viral detection from tissue samples like the kidney is generally considered most reliable, but we aimed to evaluate nonlethal swab methods enabling load detection at various time points in each individual's disease progression. Regardless of dose, inoculation method, or detection method, viral loads from sick frogs at time of death fell in a similar range (Figure S3), except tissue samples from the intraperitoneally injected group (the only significant difference in the pilot with $p < 0.05$ was between the buccal and liver detection methods in this inoculation group). While buccal swabs usually enabled slightly higher quantity detection than skin swabs, skin-buccal swab pairs performed on any given individual and time point exhibited no positive/negative mismatches (positive detection = 80 MCP gene copies). Thus, we chose to use skin swabs in the coinfection experiment because they produced comparable results to buccal swabs in the pilot, were less invasive and stressful to the animal, and allowed us to simultaneously swab for Bd.

Table S1. Pairwise comparisons of survival between treatment groups, using Log-Rank tests (function *survdif* from the *survival* package). *p*-values were corrected using the Benjamini and Hochberg's false discovery rate. Bold numbers denote $p < 0.05$.

	Bd	BdRv	Bd→Rv	Control	Rv
BdRv	1.5E-03	-	-	-	-
Bd→Rv	8.3E-01	2.7E-03	-	-	-
Control	3.3E-05	3.3E-05	3.3E-05	-	-
Rv	1.2E-04	8.1E-04	2.2E-04	1.4E-01	-
Rv→Bd	6.6E-04	9.2E-01	8.1E-04	3.3E-05	8.1E-04

Table S2. Pairwise comparisons between treatment groups based on survival curves generated by mortality events observed as days following exposure to Bd, using Log-Rank tests (function *survdiff* from the *survival* package). *p*-values were corrected using the Benjamini and Hochberg’s false discovery rate. Bold numbers denote $p < 0.05$.

	Bd	BdRv	Bd→Rv	Control
BdRv	1.8E-03	-	-	-
Bd→Rv	7.7E-01	2.7E-03	-	-
Control	2.2E-05	2.2E-05	2.2E-05	-
Rv→Bd	2.6E-03	1.2E-04	3.0E-03	2.2E-05

Table S3. Post-hoc multiple comparisons of mean log-transformed Bd load 10 days post-exposure between treatment groups using Tukey HSD test. (Significance was validated using the *glht* function in the *multcomp* package). Bold numbers denote $p < 0.05$.

Group1	Group2	Mean_Difference	Lower_CI	Upper_CI	P_Adj
BdRv	Bd	-3.033	-4.321	-1.744	4.4E-06
Bd→Rv	Bd	1.165	-0.124	2.454	8.7E-02
Rv→Bd	Bd	1.460	-0.147	3.067	8.5E-02
Bd→Rv	BdRv	4.198	2.839	5.556	3.4E-08
Rv→Bd	BdRv	4.493	2.829	6.156	4.2E-07
Rv→Bd	Bd→Rv	0.295	-1.369	1.959	9.6E-01

Table S4. *P*-values from post-hoc multiple comparisons of mean log-transformed Rv load between treatment groups using Tukey HSD test (Bd→Rv and control groups excluded from day 10 comparisons). The *p*-value appearing before the ‘/’ is from day 10 (post exposure) loads, and after the ‘/’ is from endpoint/TOD loads. The d10:end t-test column contains *p*-values from paired-end t-tests between the day 10 loads and endpoint loads within each group. ‘-’ denotes that day 10 loads were not available for a group, either because frogs died before the swab day (the case for Bd→Rv) or because swabs were not analyzed and assumed to be negative (the case for ctr).

	d10:end t-test	BdRv	Bd→Rv	Rv	Control
BdRv	0.509				
Bd→Rv	-	- / 0.989			
Rv	0.244	0.952 / 0.999	- / 0.981		
Control	-	- / 0.408	- / 0.185	- / 0.416	
Rv→Bd	0.400	0.804 / 0.997	- / 0.999	0.933 / 0.994	- / 0.266

Table S5. Post-hoc multiple comparisons of lifespan in days post exposure to Bd between treatment groups using Tukey HSD test. Bold numbers denote $p < 0.05$.

Group1	Group2	Mean_Difference	Lower_CI	Upper_CI	P_Adj
BdRv	Bd	5.300	1.566	9.034	0.003
Bd→Rv	Bd	0.175	-3.559	3.909	0.999
Rv→Bd	Bd	-4.200	-7.934	-0.466	0.023
Bd→Rv	BdRv	-5.125	-9.061	-1.189	0.007
Rv→Bd	BdRv	-9.500	-13.436	-5.564	0.000
Rv→Bd	Bd→Rv	-4.375	-8.311	-0.439	0.025

Table S6. Statistics on Bd load and lifespan for each Bd-exposed treatment group and controls. Bd load units are zoospore equivalents extracted from skin swabs. The first two columns (Day 10, Day 20) provide mean Bd load $\pm$ standard deviation detected in frogs 10 and 20 days after exposure to Bd, respectively. The TOD/endpoint column provides mean Bd load $\pm$ standard deviation detected in frogs at time of death (for all Bd-exposed frogs) or experimental endpoint (controls). The Lifespan column provides mean lifespan $\pm$ standard deviation in days following exposure to Bd. For all reported values, n = sample size available for the calculation. “-” denotes when no frogs were swabbed (died prior to swab day). A load of 0 was imputed for control frogs at day 10 and 20 (denoted with “*”): these swabs were never analyzed but assumed to be negative because control frogs were negative at day 0 and endpoint and never developed symptoms.

Treatment	Day 10	Day 20	TOD/endpoint	Lifespan
Control	0* (n = 10)	0* (n = 10)	0.08 $\pm$ 0.25 (n = 10)	34 $\pm$ 0.0 (n = 10)
Bd	1.11e04 $\pm$ 6.54e03 (n = 10)	-	8.57e04 $\pm$ 7.21e04 (n = 10)	14.2 $\pm$ 1.81 (n = 10)
BdRv	8.29e02 $\pm$ 8.34e02 (n = 8)	1.92e+04 $\pm$ 2.53e04 (n = 4)	1.64e05 $\pm$ 2.68e05 (n = 8)	19.5 $\pm$ 4.38 (n = 8)
Bd→Rv	5.17e04 $\pm$ 5.50e04 (n = 8)	-	8.45e04 $\pm$ 8.82e04 (n = 8)	14.4 $\pm$ 1.85 (n = 8)
Rv→Bd	4.75e04 $\pm$ 2.55e04 (n = 4)	-	2.27e05 $\pm$ 1.72e05 (n = 7)	10 $\pm$ 3.02 (n = 8)

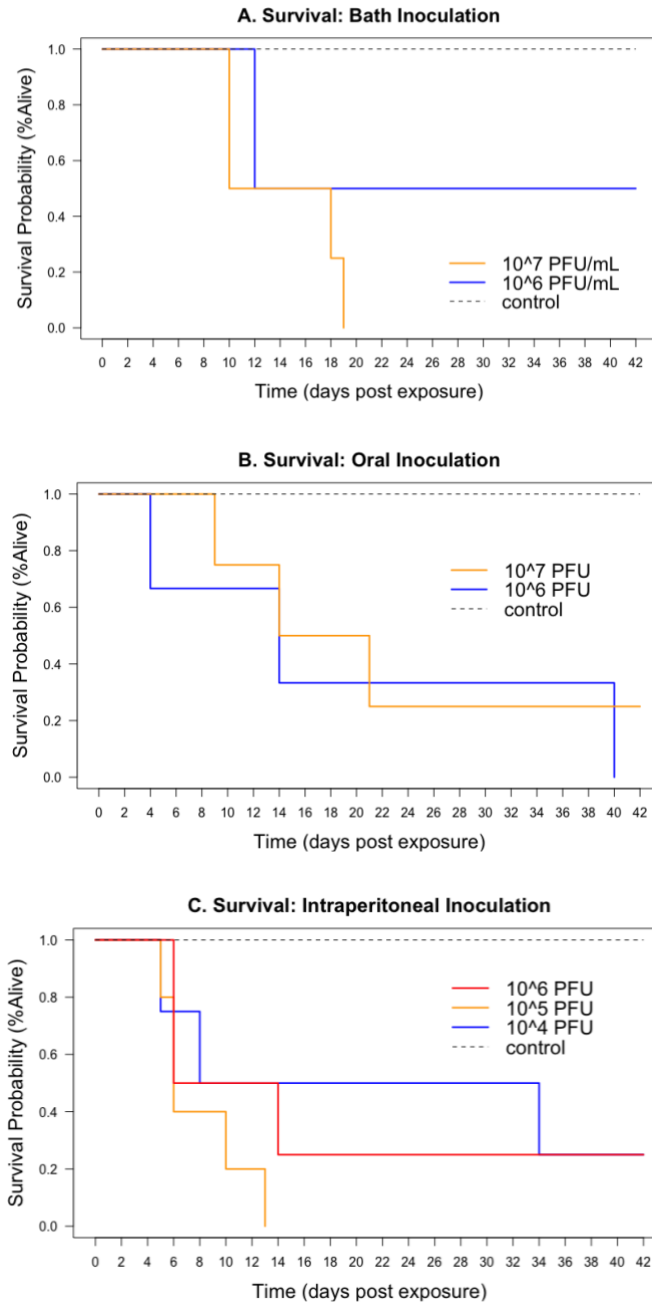


Figure S1. Survival probability curves for each treatment group in the pilot experiment. Each graph depicts survival curves constructed from frogs exposed to various doses of Rv via (A) water bath, (B) oral administration, or (C) intraperitoneal injection. PFU = plaque-forming units of Ranavirus FV3. p -value > 0.05 for all pairwise comparisons (*pairwise_survdif*) between exposed groups.

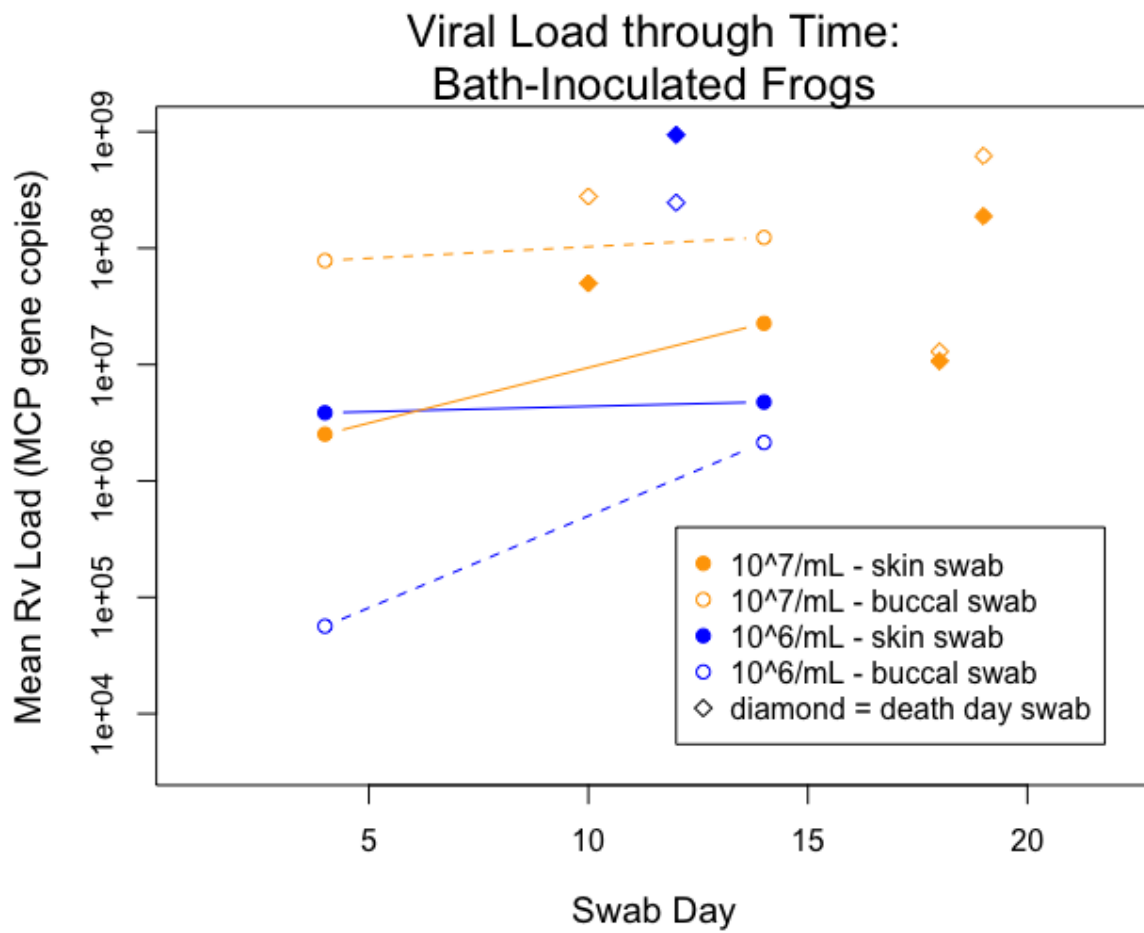


Figure S2. Rv load progression through time for water bath-inoculated frogs exposed to either 10^7 PFU/mL (orange) or 10^6 PFU/mL (blue). Lines connect the points representing mean Rv load from routine swab days 4 and 14, and free-floating diamonds represent individual loads at times of death. Hollow points and dotted lines indicate load trends detected by buccal swabs, while opaque points and solid lines indicate load trends detected by skin swabs.

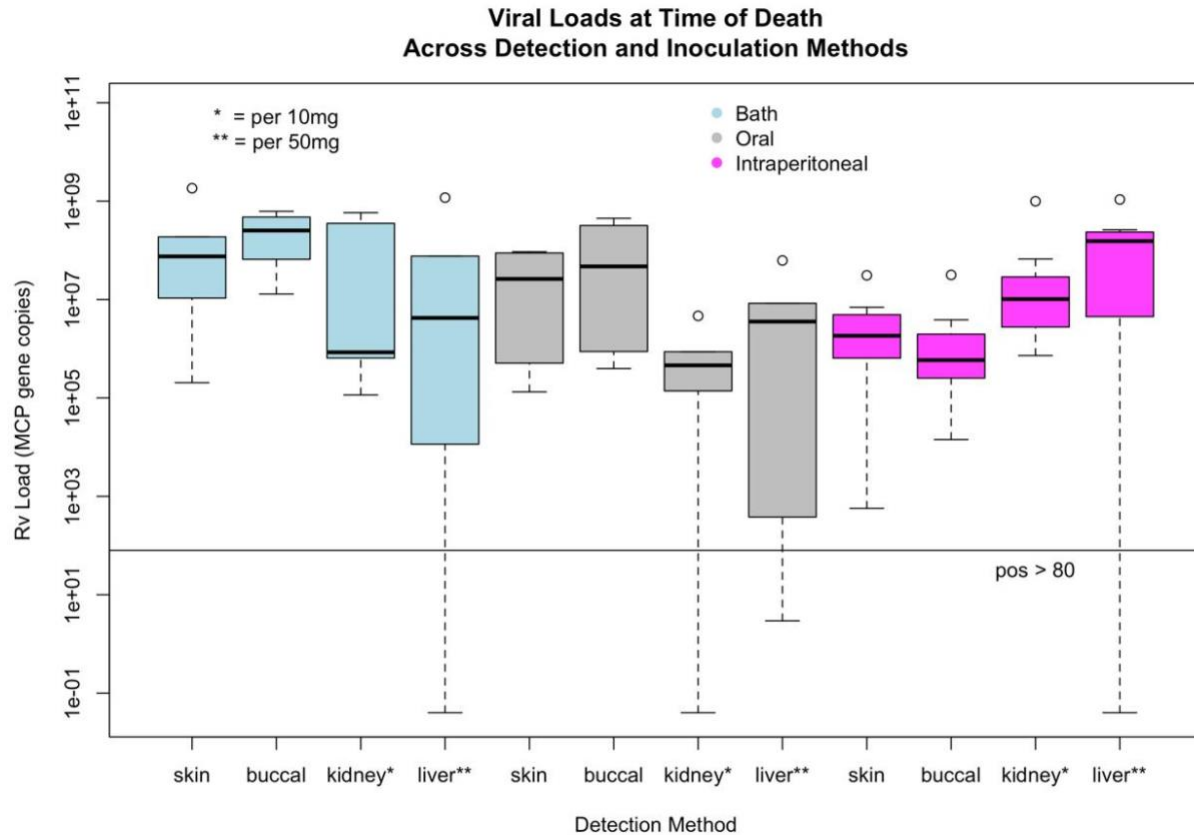


Figure S3. Standard box plots of Rv load at time of death for singly-infected frogs in the pilot experiment. Skin and buccal refer to swab methods, and kidney and liver refer to tissue extraction methods. Bath, oral, and intraperitoneal refer to inoculation methods. Because there were no appreciable within-inoculation group differences in endpoint Rv loads between doses, we pooled load data from all doses within each inoculation group.

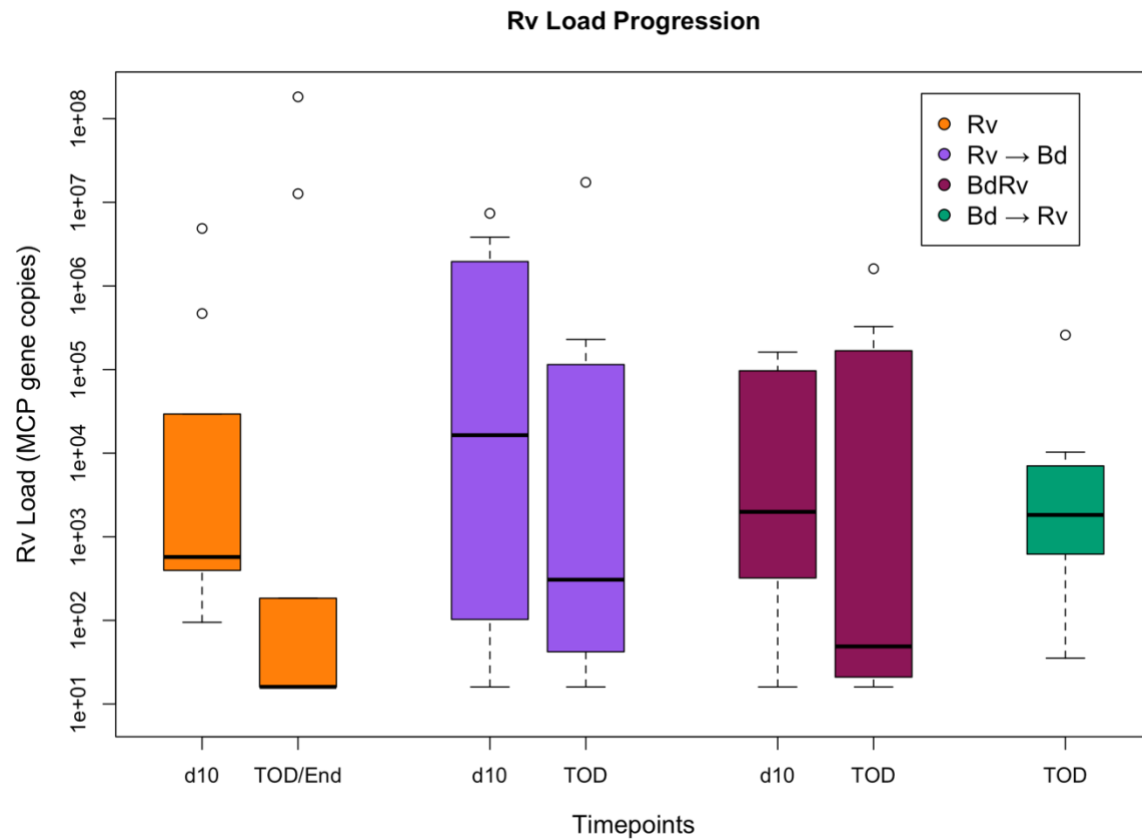


Figure S4. Standard box plots of log-transformed Rv load detected on individual skin swabs for each treatment group, performed 10 days after Rv exposure (d10) and at time of death and experimental endpoint (TOD/End). Note we display no day 10 load for the Bd→Rv group because no individuals survived 10 days after Rv exposure. The two displayed outliers at TOD in the Rv-only group (orange) correspond to the only mortalities in this group. Tukey's HSD post-hoc tests and paired t-tests revealed no significant differences in Rv load between any two groups or time points ($p > 0.05$, Table S4).

References

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