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Investigation of Prevalence of Co-Infection by *Batrachochytrium dendrobatidis* and Ranavirus in Endemic Beyşehir Frog (*Pelophylax caralitanus*)

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Abstract

Batrachochytrium dendrobatidis (Bd) and Ranavirus (Rv) are among the pathogens responsible for the rapid decline of amphibian populations worldwide. The aim of this study is to determine the presence of both pathogens in the endemic Beyşehir frog population living in the Lakes Region in the southwestern Anatolia between 2014-2015 years. Prevalences of each pathogen and co-infections (i.e., presence infection with both of Bd and Rv in same animal) were 48.6 %, 10.3 % and 7.0 % respectively and differences in prevalences were detected among lakes and years. This study is the first report of Rv infection and co-infection with Bd and Rv of endemic Beyşehir frog (*Pelophylax caralitanus)* in Turkey. Although there are infection by both pathogens, it is unclear that how effective in declining of *P.caralitanus* population.

Keywords: *Batrachochytrium dendrobatidis*, Ranavirus, *Pelophylax caralitanus*, Co-Infection

Endemik Beyşehir Kurbağası (*Pelophylax caralitanus)*'nda *Batrachochytrium dendrobatidis* ve Ranavirus ile Ko-enfeksiyon Yaygınlığının Araştırılması

Özet

Batrachochytrium dendrobatidis (Bd) ve Ranavirus (Rv), dünya çapında amfibi populasyonlarının hızla azalmasından sorumlu patojenler arasındadır. Bu çalışmanın amacı, 2014-2015 yılları arasında Güneybatı Anadolu Bölgesi'ndeki Göller Bölgesi'nde yaşayan endemik Beyşehir kurbağa populasyonunda her iki patojenin varlığını saptamaktır. Her bir patojen ve koenfeksiyon varlığı sırasıyla %48,6, % 10,3 ve % 7,0 oranında tespit edildi ve göllerin ve yıllar arasında yaygınlık oranlarında farklılıkları tespit edilmiştir. Bu çalışma, Türkiye'de endemik Beyşehir kurbağası (*Pelophylax caralitanus)*'nda Bd ve Rv'si ile Rv enfeksiyonu ve ko-enfeksiyonunun ilk rapordur. Her iki patojen tarafından da enfeksiyon olmasına rağmen, *P.caralitanus* populasyonunun azalmasında ne kadar etkili olduğu tespit edilmemiştir.

Anahtar kelimeler: *Batrachochytrium dendrobatidis*, Ranavirus, *Pelophylax caralitanus*, Ko-enfeksiyon

INTRODUCTION

Several factors such as habitat loss, deforestation, excessive collection, touristic activities, predator species, agricultural chemicals, domestic waste, environmental pollution, disease and global climate change have been shown to explain the declines in amphibian population worldwide (Blaustein and Wake, 1995; Schock et al., 2010). Many factors can associate with the decline in global amphibian population for instance the destruction of habitats, increase in ultraviolet radiation, rise in the number of predatory species and the incidence of infectious diseases (Blaustein and Wake, 1995; Alford and Richards, 1999; Gardner et al., 2005; Beebee and Griffiths, 2005).

A rise in the incidence of some infectious disease such as saprolegniasis, chytridiomycosis and Ranavirus has a causative role in the mortality and morbidity of amphibian populations (Daszak et al., 1999; 2003; Kilpatrick et al., 2010).

Chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (Bd), is an infectious amphibian disease that has been associated with the decline in population worldwide (Berger et al., 1998; Laurance, 2008). Bd infects amphibians from the superficial, keratin-containing skin layers. (Berger et al., 1998; Rachowicz and Vredenburg, 2004). Infected frogs begin to die about 21 day, death usually occurs in adult animals (Voyles et al., 2012). Recent studies in Turkey have shown that Bd is present in *Pelophylax ridibundus*, *Hyla orientalis*, *Pseudepidalea variabilis*, *P. caralitanus* and *P. bedriagae* (Gocmen et al., 2013; Erismis et al., 2014).

Iridoviridae family include Ranavirus (Rv) genus with other four genera, which are Iridovirus, Lymphocystivirus, Chloriridovirus and Megalocytivirus. Lymphocystiviruses and Megalocytiviruses infect fishes, Iridoviruses and Chloriridovirus infect insects and Rv viruses responsible for mortality and morbidity in amphibians, fishes and reptiles (Chinchar, 2002).

Many ectothermic vertebrates such as amphibians, fishes and reptiles were infected by Rv which detected from many diseased or healthy vertebrates as well as in invertebrates in Australia, America, Europa and Asia. Global amphibian declines have adverse effect on economy and ecology (Fijan et al., 1999; Speare and Smith, 1992; Chinchar et al., 2002; 2009; Hyatt et al., 2002). Infection can be transmited by animals ingesting other infected animals and contracting of animal-to-animal (Cullen and Owens, 2002; Picco and Collins, 2008). Furthermore, viruses spread by the translocation of infected fishes and the equipment of fishers such as boats, nets, and baits. (Pico et al., 2007). In addition, birds being potential vectors, can carry Rv on their feathers, bill and feet from one geographical location to others (Whittington et al., 1996).

P. caralitanus, the endemic Beyşehir frog, has been recently described species (Arikan, 1988) in the Lake District of southwestern Anatolia. Historically, it has been a common inhabitant of lakes and ponds at elevations of 950 - 1200 m (Arıkan, 1988; Budak et al., 2000; Jdeidi et al., 2001), ranging from the Konya plain to Denizli. Habitat loss as well as over-harvesting by commercial collectors principally for the Western European frog-leg market poses potential ongoing threats to the survival of this species. While it remains locally abundant at some sites, overall the Beyşehir frog population is in rapid decline and is now considered an endangered species [(IUCN International Union for Conservation of Nature)-(the IUCN Red List of threatened Species - 2010)].

Recently studies have shown that Chytridiomycosis is one of the threatening factor for amphibian in Turkey (Gocmen et al., 2013; Erismis et al., 2014), although there are a few studies about chytridiomycosis, there is not any report on Rv infection. To our knowledge, there has not been any published data dealing with the co-occurrence of these two pathogens in amphibian populations in Turkey. The aim of this study was then to estimate the prevalances of Bd and Rv in *P. caralitanus* in Lakes between May-2014 and July-2015.

MATERIALS and METHODS

Field Sampling

A total of 214 individuals (114 in 2014 and 100 in 2015) of Endemic Beyşehir frogs (*P. caralitanus*) were ramdomly collected from Işıklı Lake, Beyşehir Lake and Eğirdir Lake where located in Lake District, Turkey (Figure 1).

We used standardized sampling protocol for *Bd* detection (Hyatt et al., 2007; Marantelli et al., 2004; Kriger and Hero, 2007). Animals were swabbed 30 times along keratinized surfaces with sterile swabs (Medical Wire Company MW113) and swabs were stored in 95 % ethanol. Each animal was placed into an individual sterile bag containing 1 cm deep fresh-water for a while. Then, we collected skin rashes into a container. The sample of skin rashes were then placed in 2.0 ml sterile centrifuge tubes containing 95 % ethanol (Souza et al., 2012). At the end of sampling, animals were released to their own habitats. Collected samples were stored at -80 °C until analysis.

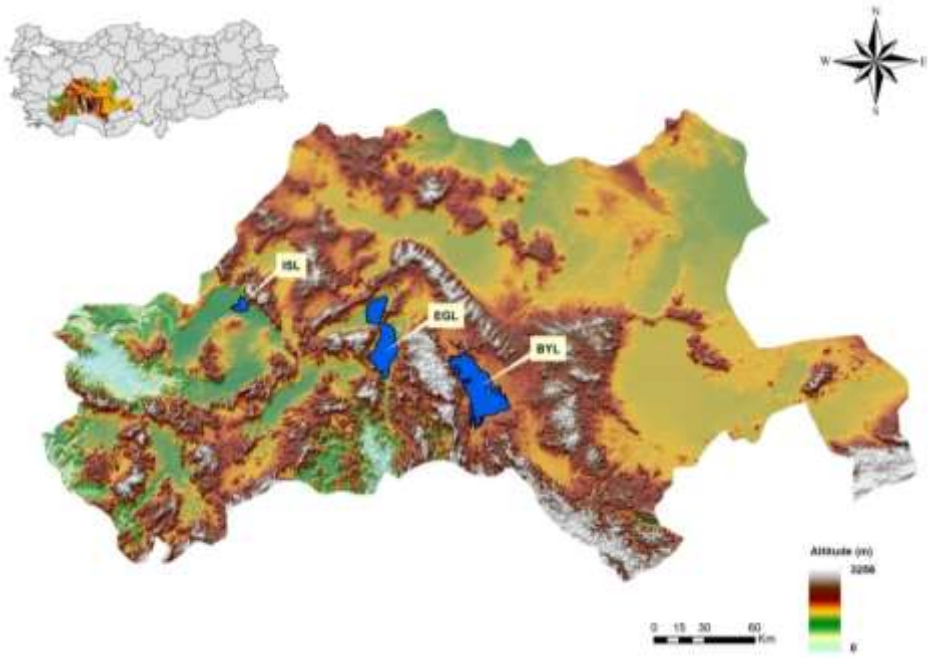

Figure 1. Location of the four study sites in the Lakes District of southwestern Anatolia, Turkey. Locality abbreviations: BYL, Beyşehir Lake; EGL, Eğirdir Lake, and ISL, Işıklı Lake

Sample Analysis

For *Bd* DNA isolation, 30-40 mg of 0.5 mm diameter zirconium / silica beads were placed in the tubes. After adding an aliquot's of 50 µl PrepMan Ultra (Applied Biosystems # 4318930), the swaps were added into the tubes. The samples in the tubes were then homogenized at 4000 rpm for 45-60 sec with a BeadBug (Benchmark) device that allows for the disintegration of existing zoospores. After incubation for a short time period in the ice cube followed by centrifugation at 13,000 x g for 30 sec, the homogenization and centrifugation were repeated. The covers of the tubes were pierced using sterile needles of No. 22 or 23 gauge, and the tubes were kept in boiling water-bath for 10 minutes. The samples taken from the water bath were allowed to cool down at room temperature for 2 minutes, followed by 3 minutes at the highest speed (~ 13,000 x g) centrifugation at +4 °C. Supernatant was diluted 10 fold (~ 5 ng/µl) to use in qPCR procedures. The amount of DNA was measured by using fluorometric Qbit 2.0 device (Invitrogen tech.) and DNA samples were stored at -20 °C until analysis (Kriger et al., 2006).

For Rv, the skin piece is fixed in an microcentrifuge tube containing 95% ethanol. Fifty mg skin was transferred into 400 µl lysis buffer (NaCl 0,1 M, sucrose 0.5 M, Tris 0.1 M, EDTA 50 mM, SDS 0.5%) and homogenized with a homogenizer. The resulting homogenate was incubated for 30 minutes at 65 ° C. Then 57 µl of 8M potassium acetate was added on homogenate and incubated for 30 min in an ice bath. After incubation, the samples were centrifuged at 10,000 g for 15 min and the supernatant was transferred into a new microtube. One ml of ice-cold ethanol was added on samples. The DNA was then precipitated by centrifugation at 10,000 g for 15 min and stored at + 4 ° C and then at -20 ° C by dissolving in 50 µl of TNE (Tris 6mM, NaCl 6mM, EDTA 0.2mM) buffer (Galli et al. 2006).

We used qPCR protocols previously described by Boyle et al., 2004; Kriger et al., 2006; Galli et al., 2006; Hyatt et al., 2007. For Bd, we used 20 µl reaction buffer with 5 µl template DNA and 25 µl reaction buffer with 5 µl of DNA template. Bd qPCR reaction buffer included 10 µl 2x Precision™ MasterMix (Genesig Advanced Kit, United Kingdom,), 1 µl of 10 picomole primer/probe mix (Genesig Advanced Kit, United Kingdom) and 4 µl nuclease-free water. Plates were runned at 1 cycle 95 °C (10 m.) and 50 cycles 95 °C (10 s), 60 °C (60 s).

We used Rv-specific MCP (Major Capsid Protein) and IE (Immediately Early) primer sets (Galli et al., 2006). We ran Rv reactions 10 µl SYBR Green Master Mix (Applied Biosystems), 1 µl 10 picomole forward primer and reverse primer and 8 µl nuclease-free water. Samples for both pathogens were runned in triplicate on an Exicycler™ 96 (Bioneer) Real Time PCR machine. Each plate contained negative controls. Bd analysis plates contained standards as the internal positive

controls. The positive controls and the standard curves were constructed using 10-fold for the component of serial dilutions kit (Genesig Advanced Kit, United Kingdom). For Rv testing, PCR products were checked via electrophoresis on a 1.0 % agarose gel (Galli et al., 2006).

Statistical Analysis

In Bd analysis, swabs were considered as positive when $G_e>0$ and negative when $G_e=0$. In RV analysis, samples were determined to be positive with qPCR $C_t<35$. The prevalence of infection for both pathogens and co-infection e with 95% confidence intervals was calculated by dividing infected samples by total specimens. The chi-square test was used to determine differences in the prevalence of pathogens and co-infection between years and lakes. All analyses were performed using SPSS Statistic (IBM, version 23) software at $P<0.05$.

RESULTS

We sampled 214 *P. caralitanus* over two years. 114 samples were collected during 2014 and 100 samples were collected during 2015. The overall prevalences of infection of *Bd, Rv,* and coinfection were 48.6 % (41.7-55.5; % 95 CI), 10.3 % (6.6-15.2; 95 % CI) and 7.0 % (4.0-11.3; % 95 CI), respectively. In EGL Lake, there is no positive result for Rv in 2014. In addition, in the same year, there was no co-infection in any lake (Table 1).

The prevalence of *Bd* infection was grater in 2015 than in 2014 all lakes and total prevalence was significantly increased from 34.2 % to 65.0 % ($P<0.001$). The prevalence of infection by *Bd* was the highest in ISL Lake in 2014 and 2015 years ($P=0.001$). On the other hand, EGL Lake showed the lowest prevalence in both of years. Intensity of *Bd* infection calculated using by standard curve (ranged from 10^1 to 10^6). In BYL lake, 40 % of individuals had *Bd* infection intensity above 1000 zoospore genomic equivalent (G_e) in 2014 and the individuals in 2015 had not shown above 1000 G_e. In EGL lake, the 12.5 % of samples had *Bd* intensity above 10.000 G_e in 2014. In addition, the samples collected in 2015 had *Bd* intensity below 1000 G_e. In the ISL Lake, the 20 % of individuals had infection intensity above 1000 G_e.

We found significant differences in total prevalence of Rv infection among years ($P<0.001$), but we did not find among the lakes ($P>0.05$). Prevalence of Rv increased in all lakes from 2014 to 2015. Although no Rv infection was detected in EGL Lake in 2014 year, a significant increase was observed in 2015. Additionally BYL Lake has the highest infection ratio in both of 2014 and 2015 years (Table 1).

In 2014 year, both Bd and Rv infections could not be found, but in 2015, coinfection was found in all places by 15.0 % coinfection prevalence ($P<0.001$). ISL Lake, which has the highest values in the prevalence of Bd infection in 2014 and 2015, has the highest value in the prevalence of coinfection (Table 1).

Table 1. Prevalence with 95% confidence interval of *Batrachochytrium dendrobatidis* (*Bd*), Ranavirus (Rv), and coinfection by year and in endemic Beyşehir Frog (*Pelophylax caralitanus*) of Turkey. BYL: Beyşehir Lake, EGL: Eğirdir Lake, ISL: Işıklı Lake

	Lake	ºC	H %	+Bd	Bd prevalance (CI)	+Rv	Rv prevalance (CI)	Co-infected	Co-infection prevalance (CI)	Total (N)
May2014	BYL	21	38	10	37.0 %(19.4 - 57.6 %)	2	7.4 %(0.9 - 24.3 %)	0	-	27
	EGL	21	42	16	30.2 %(18.3 - 44.3 %)	0	0	0	-	53
	ISL	23	44	13	38.2 %(22.2 - 56.4 %)	2	5.9 %(0.7 - 19.7 %)	0	-	34
July2015	BYL	34	44	26	74.3 %(56.7 - 87-.5 %)	7	20 %(8.4 - 36.9 %)	4	11.4 %(3.2 - 26.7 %)	35
	EGL	38	52	9	34.6 %(17.2 - 55.7 %)	4	15.4 %(4.4 - 34.9 %)	4	15.4 %(4.4 - 34.9 %)	26
	ISL	35	46	30	76.9 %(60.7 - 88.9 %)	7	17.9 %(7.5 - 33.5 %)	7	17.9 %(7.5 - 33.5 %)	39
	Total			104	48.6 %(41.7 - 55.5 %)	22	10.3 %(6.6 - 15.2 %)	15	7.0 %(4.0 - 11.3 %)	214

DISCUSSION

Bd infection and co-infection prevalence were the highest in ISL lakes. The density of the animals studied in fieldwork was also the highest in ISL lakes too. We think that the high density of population accelerates the spread of infections. However, this hypothesis needs to be further supported through future works on the endemic *P. caralitanus* species in the lakes area. Despite the abundance of frog population in the EGL Lake, the rate of infection does not change significantly over the years. This might be due to the high flowing rate in the sampling regions of lakes since the high rate of water flow prevents zoospores infection to other animals and therefore, prevents spreading the co-infection of multiple pathogens within amphibian hosts. It has been reported that the co-occurrence of Bd and Rv within sites (though not within hosts) (Fox et al., 2006; Hoverman et al., 2012; Souza et al., 2012). Furthermore, Miller et al. (2008) documented the concurrent infections of Bd and Rv in captive amphibians. For example, Miller et al. (2011) summarized the information on Rv infection in amphibians and demonstrated infections in 9 of 70 species of amphibians from 14 families. In a previous study in this area, the only prevalence of *Bd* in BYL, EGL and ISL lakes were 32.1 %, 14.0 % and 20.5 %, respectively (Erismis et al., 2014) and to our knowledge, there was not any studies aimed to document the co-infections by Bd and Rv in Turkish amphibians. In this study, the presence of Rv and co-infection in the frog was initially studied along with the detection of increase in *Bd* infections. Furthermore, our current study presents the first data about the known Rv infection and co-infection with Bd and Rv in the wild populations of endemic Beyşehir frogs. The prevalence of Rv infection has increased at least twice as much in each lake in 2015 in comparison to 2014. Especially in EGL Lake, although the infection was not encountered in 2014, 15.4% was detected in 2015. The prevalence of Rv infection in BYL and ISL lakes was found to be increased significantly in 2015 year in comparison to 2014 year, and a higher prevalence was detected in the BYL in the same year analysis. While no co-infection was found in the samples in 2014, 15 animals were found to be infected with both *Bd* and Rv in the 2015 samples. The prevalence of coinfection in ISL Lake was found to be higher than in the BYL and EGL lakes. And also, the prevalence of *Bd* infection in ISL lake was higher than other lakes. Failure to identify Rv in 2014 can depend on the sampling season and area. Our findings suggest that the intensity of populations and a stationary smaller water environment reveal that the spread of infections occur more easily and rapidly. Moreover, the ISL Lake has a lower altitude than other lakes and the milder climatic conditions provide a more favorable environment for the development of pathogens. The temperature and humidity recording in the three showed that the prevalence and the severity of disease outbreaks were greater during the warmer months (Table 1). Our finding support the previous studies about the relationships with climate change and the epidemiological effects of both pathogens on amphibians (Fox et al., 2006; Miller et al., 2007; Rothermel et al., 2008; Schock et al., 2010; Reshetnikov et al., 2014; Warne et al., 2016; Frances et al., 2016).

Rv and co-infection prevalance is present in each three lakes at very low prevalence rates. Despite the high prevalence of Bd sampled in all three lakes, there was not one die off event observed during our sampling. There is the possibility that mortality events can be occurred in active period of the animal after the sampling events at May - 2014 and at June 2015. Given that mortality rates closely track infection rates (Brunner et al., 2007; Cunningham et al., 2007; Hoverman et al., 2011), additional mortality events likely can be occurred before/after our study but were undetected. Another study starting in 2010 was stressed that a steep enigmatic decline was observed in an endangered but stable population of fire salamanders (*Salamandra salamandra*) in the south of the Netherlands. In 2015, only 4% of the population remained. None of the mortalities could be attributed to Bd or any other known viral or bacterial amphibian pathogen (Spitzen-van der Sluijs et al. 2013). This is the first study of determined in Rv infection and co-infection in Turkey.

Our finding in three lakes with Bd, Rv and co-infection prevalence levels suggests that die offs may have been imminent but missed due to a single visit in that season, therefore our results underscore the need for intensive monitoring over time to assess the impacts of co-infection on amphibian populations. Because the increased prevalence of pathogens over the years suggests that similar studies and monitoring of population in the region should continue to be studied through earlier season sampling and later season sampling in the year. Field trials and controlled condition studies will help to reveal the role of epidemic diseases that threaten endemic Beyşehir frog population

and remaining other Turkish amphibian species. Investigation of other pathogens as well as Bd and Rv will be of a great importance for the protection of the aquatic ecosystem in the region.

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