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Original article

Investigation of *Salmonella* spp. and *Escherichia coli* in the Snake-eyed lizard (*Ophisops elegans)* (Sauria, Lacertidae) in the Çankırı Province of Turkey

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Abstract

Zoonoses are frequently associated with wild animals. Research on reptiles either living in their natural habitat or kept as pet animals has shown that these animals frequently serve as the asymptomatic hosts of bacterial zoonotic agents, including *Salmonella* spp. and *Escherichia coli*. Studies have shown the potential of reptiles to transmit these pathogens to humans and other animals. Epidemiological research on the herpetofauna of various regions has demonstrated the high potential of reptiles as a reservoir of *Salmonella* spp. In the present study, *Salmonella* spp. were not isolated or identified from the snake-eyed lizard. Out of 150 cloacal swab samples of snake-eyed lizard 25 (16.7%) *E. coli* were isolated and out of these 4 (2.7%) were identified to be *E. coli* O157:H7 by PCR. The results suggest that *Ophisops elegans* could be involved in the transmission of *E. coli*, rather than *Salmonella* spp. This study demonstrates for the first time that the snake-eyed lizard acts as a cloacal carrier of *E. coli* O157:H7 and presents data that may aid in preventing the transmission of this strain to humans.

Key words: bacterial infection, PCR, reptilia, Turkey

Introduction

Zoonoses are frequently associated with wildlife and pose a threat to public health. Thus, the investigation of zoonoses in wild animals is a topic that still retains its significance today (Allen et al. 2017). Zoonoses have an important place in the emergence of contagious diseases, and the majority are associated with livestock and with wild animals and domestic pets. Disease outbreaks mostly occur as a result of humans directly contacting these animals, being bitten by them, consuming either exceptionally or recreationally their products, and by consuming food contaminated with their secretions or excretions (Bender and Shulman 2004).

Salmonella spp. and *Escherichia coli* are bacteria capable of colonizing the gut of various animals, and

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Fig. 1. Collection of cloacal swab samples from the snake-eyed lizard *Ophisops elegans.*

thus may threaten human and animal health. These bacterial pathogens have been isolated and identified from multiple avian and mammalian species that serve as their host (Şahan et al. 2016, Gambi et al. 2022, Hawwas et al. 2022, Babacan 2023). While the presence of *S. enterica* serovars is frequently associated with diseases, *E. coli* serovars are generally accepted as commensal organisms (Lupolova et al. 2017). However, among these serovars, *E. coli* O157:H7 has been reported to cause multiple diseases including the hemolytic uremic syndrome (HUS), hemorrhagic colitis and thrombotic thrombocytopenic purpura (TTP) (Engdaw and Temesgen 2016).

Recent studies have reported the association of *Salmonella* spp. and *E. coli* outbreaks in humans with reptiles. Of all human salmonellosis cases, 3 to 5% have been diagnosed in people known to be in contact with reptiles, and these cases are considered to have reptilian origin and called as Reptile-Associated Salmonellosis (Wooley et al. 2001, Spickler 2013). The snake-eyed lizard *Ophisops elegans* has been reported to be distributed within an area ranging from southern and western Europe, Turkey and northern and eastern Asia to northern Africa (Oraie et al. 2014). Reptiles are hunted by various raptors, some predatory mammals and poultry, and therefore, the investigation of the pathogens carried by these animals bears significance with respect to protecting the health of wild animals, humans and domestic animals (Bakaloudis et al. 2012, Nielsen and Bull 2016). To date, many studies have demonstrated bacterial agents including *Salmonella* spp., *E. coli* and *Clostridium* spp. to be carried by reptiles showing no disease symptom (Ramos et al. 2019).

This study was aimed at the investigation of the presence of *Salmonella* spp. and *E. coli* in the snake-eyed lizard *O. elegans*, which inhabits the Çankırı province and its vicinity in Türkiye, by analyzing cloacal swab samples with the culture and polymerase chain reaction (PCR) methods.

Materials and Methods

Study material and samples

This study was conducted pursuant to the 2020/102 numbered approval of the Local Ethics Board for Animal Experiments (HADYEK) of Kafkas University and the E-21264211-288.04 numbered permission of the General Directorate of Nature Conservation and National Parks of the Ministry of Agriculture and Forestry of the Republic of Turkey.

The study material comprised of cloacal swab samples taken from 150 live snake-eyed lizard (*O. elegans*) captured manually (Eekhout 2010) in the vicinity of the Çankırı province of Turkey (40°31′47″ N, 33°35′57″ E). The lizards were captured from 10 different locations, 10 to 20 lizards from each village, hosted in a plastic bucket for 30 min until sampling and released just after the sampling. Cloacal samples taken manually with cotton swabs from the captured lizards were transferred into screw-capped glass tubes containing 500 µl of physiological saline solution. The samples were transported to the Central Laboratory of Çankırı Karatekin University under cold-chain conditions and stored at -80 °C until analysis (Fig. 1).

Bacterial isolation and identification

Pre-enrichment

The samples preserved in physiological saline solution at -80 °C were first defrosted at room temperature and then homogenized on a Vortex mixer. Aliquots of 250 µl were taken from each sample tube and trans-

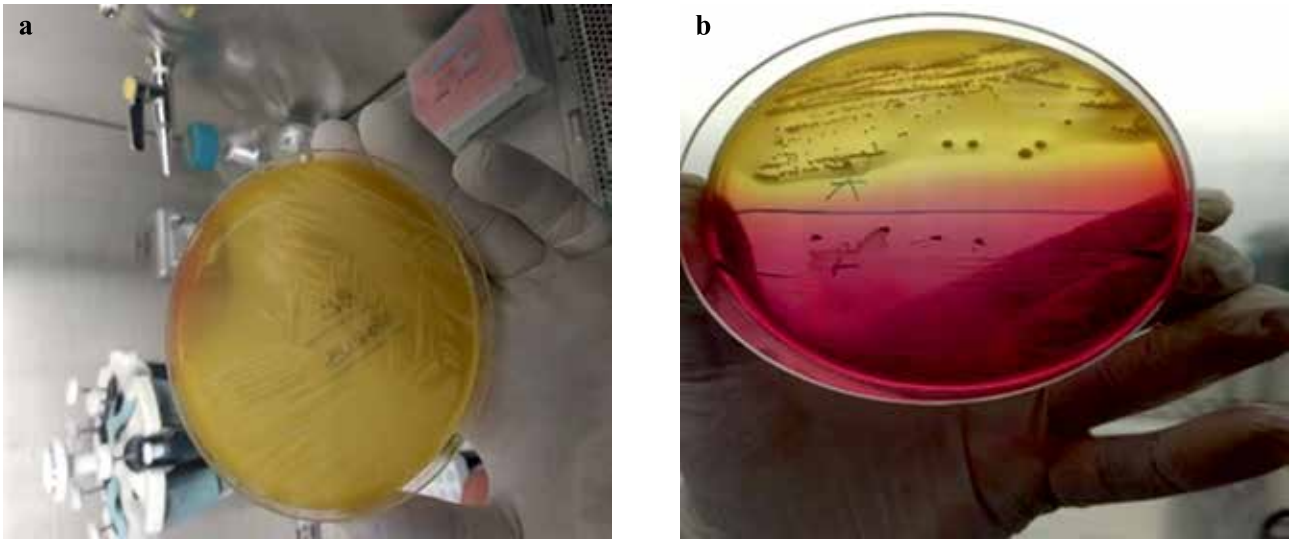

Fig. 2. Culture result images of the samples: a) xylose lysine deoxycholate (XLD) agar culture-positive for *Escherichia coli*; b) Color differences caused in XLD agar by growth of the control strains of *Salmonella typhi* and *E. coli*.

ferred into tubes containing 1 ml of buffered peptone water to be incubated at 38°C under aerobic conditions for 24 hours.

Enrichment

After being pre-enriched in buffered peptone water, the samples were inoculated in a volume of one-fifth of each tube into 5 ml of Rappaport Vassiliadis (RVS) broth containing 20 mg/L of novobiocin (Oxoid SR0181) and incubated at 42 °C under aerobic conditions for 3 days. One-ml samples taken from the incubated RVS broth were inoculated onto xylose lysine deoxycholate (XLD) agar (ThermoFisher Scientific, USA) and triple sugar iron (TSI) agar (ThermoFisher Scientific, USA) to undergo a 24 h-incubation period at 38°C under aerobic conditions. Colonies observed to have grown on XLD agar, which were black in the center and red-pink colored in the periphery, as well as colonies that turned the color of TSI agar to black were considered to be *Salmonella* spp., whilst yellow--colored colonies were considered to be *E. coli* (Fig. 2) (Hanson et al. 2003, Kar et al. 2017).

Molecular analysis

DNA Isolation

DNA isolation from the cultures and directly from the isolates was performed using the phenol-chloroform--isoamyl alcohol method (Trochimchuk et al. 2003).

Positive Control Strains

Salmonella spp. (*S.* Typhimurium ATCC13311) and *E. coli* O157:H7 EDL931 were used as positive control strains.

PCR Conditions

DNA samples isolated directly from the samples and from the cultures were analyzed with the PCR for *Salmonella* spp. using the F (5'-GTGAAATTATCGC CACGTTCGGGCAA-3') and R (5'-GTGAAATTATC GCCACGTTCGGGCAA-3') primers targeting the amplification of the *inv A* gene (Rahn et al. 1992). PCR for the presence of *E. coli* O157:H7 was performed using the F (5'-GTAGGGAAGCGAACAGAG-3') and R (5'-AAGCTCCGTGTGCCTGAA-3') primers targeting the *hly A* gene (Wang et al. 1997). The mixture and thermal condition for each PCR is shown in Table 2. All PCR reagents were obtained from Sigma Aldrich (USA) and Sentebiolabs (TR) unless otherwise stated in the text. PCR products were analyzed by horizontal gel electrophoresis on 1.5% agarose gel and the product with 284 bp and 361 bp was considered to be *Salmonella* spp. and *E. coli* O157:H7, respectively.

Results

Following the culture of 150 cloacal swab samples taken from *O. elegans*, 25 (16.7%) produced yellow colonies on XLD agar and rendered a yellow color with no blackness on TSI agar, both of which were considered as proof of the presence of *E. coli*. Following culture analysis, *Salmonella* spp. were not isolated from any of the cloacal swab samples. Similarly, no positivity was detected for *Salmonella* spp. using direct PCR analysis of the cloacal swab samples. Out of the 25 isolates (16.7%) identified as *E. coli* by culture analysis, 4 (2.7%) were identified as *E. coli* O157:H7 by PCR analysis using primers specific to the *hyl* A gene

Table 1. Test findings used in the present study.

	Number of cloacal swab samples	Positive culture results	Positive direct PCR results	PCR results of the culture-positive samples
Salmonella spp.	150	0	0	0
E. coli	150	25	-	-
E. coli O157:H7	150	4	4	4

Table 2. PCR details used for the identification of *Salmonella* spp. and *E. coli* O157:H7.

Species	Primer	Sequence (5'–3')	Target gene	PCR component* (25 µl)	Thermal cycle	Amplicon (bp)	Reference
Salmonella spp.	F R	GTGAAATTATCGCCACGTTCGGGCAA GTGAAATTATCGCCACGTTCGGGCAA	*inv A*	• 2.5 µl PCR buffer (x10, with $MgCl_2$) • 0.5 dNTP (10 mM) • 1 µl primer F (10 pmol) • 1 µl primer R (10 pmol) • 0.5 µl Taq DNA polymerase (5 U) • 2.5 µl template DNA (40-80 ng/µl) • 17 µl Nuclease free water	*One cycle:* • Denaturation at 94°C for 3 min *Thirty-five cycles:* • Denaturation at 94°C for 30 sec • Annealing at 55°C for 30 sec • Elongation at 72°C for 1.5 min *One cycle:* • Final elongation at 72°C for 5 min	284	Rahn et al. 1992
E. coli O157:H7	F R	GTAGGGAAGCGAACAGAG AAGCTCCGTGTGCCTGAA	*hly A*		*One cycle:* • Denaturation at 94°C for 3 min *Thirty-five cycles:* • Denaturation at 94°C for 1 min • Annealing at 52°C for 1 min • Elongation at 74°C for 2 min *One cycle:* • Final elongation at 74°C for 10 min	361	Wang et al. 1997

* Taq DNA polymerase with 10X PCR reaction buffer containing $MgCl_2$ (D1806, Sigma Aldrich, USA), dNTP mix (71004-M, Sigma Aldrich, USA), Primer (Sentebiolab, TR), Nuclease free water (W4502, Sigma Aldrich, USA)

(Table 1). The direct PCR analysis of the cloacal swab samples with *hyl* A primers demonstrated positivity for 4 (2.7%) samples (Fig. 3).

Discussion

Zoonotic diseases may be of contagious or non-contagious nature and their causative agents can be transmitted to humans by vertebrates either directly by contact or indirectly by means of the intake of contaminated food and water or by vectors (İnci et al. 2018).

Previous research has shown that reptiles may serve as reservoirs for various bacterial pathogens, which may have led to the self-protection reactions of humans being established as permanent reflexes over time (Ebani 2017). Lizards living near human settlements or agricultural areas can acquire *E. coli* and *Salmonella* spp. Monitoring of antibiotic resistance as an indirect indicator of human effects on bacterial exposures in the wild indicates this (Thaller et al. 2010, Wheeler et al. 2012).

Cases of salmonellosis caused by *Salmonella* spp. continue to be a major health problem affecting the global human population, such that nearly 1.3 million people are reported to die from this disease annually (Pui et al. 2011). Today, while humans keep various animals, including cats, dogs, rodents, amphibians and reptiles as pets, it is reported that these animals shed *Salmonella* spp. into the environment via their feces, and thereby threaten human health (Dróżdż et al. 2021).

Reptiles, which are part of the herpetofauna, have been frequently associated with *S. enterica*. While reptiles have been reported to serve as potential reservoirs for the transmission of *S. enterica* serovars to humans (Mermin et al. 2004), very little is known

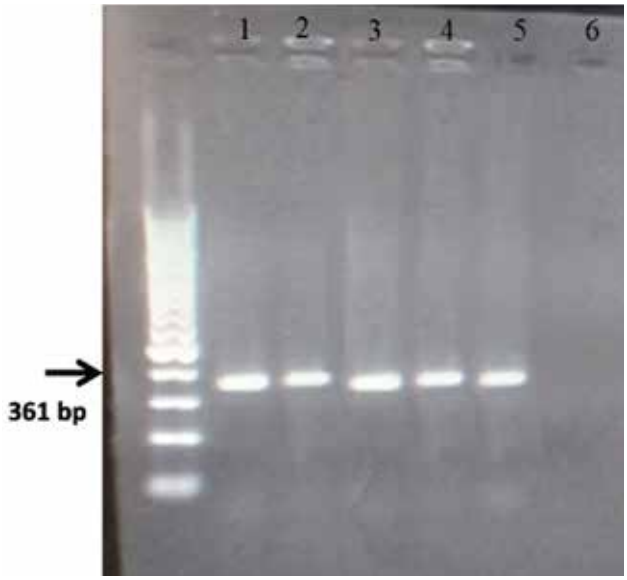

Fig. 3. mage of agarose gel electrophoresis of *E. coli* O157:H7 specific PCR. 1 is the positive control, 2, 3, 4 and 5 are positive samples for *E. coli* O157:H7 and 6 is the negative control.

about the incidence of this pathogen in reptilian species (Chambers et al. 2006). Upon reviewing the literature, microbiological studies on the herpetofauna were encountered and a very few studies were encountered on reptiles in their natural habitat (Zajac et al. 2016), but no previous literature report on *O. elegans* was come upon. In a study conducted by Middleton et al. (2010) in New Zealand, the presence of *Salmonella* spp. was investigated by the culture method in cloacal samples taken from 703 lizards living in 6 different habitats. Not having determined any statistically significant difference between the study locations for the presence of *Salmonella* spp., the researchers detected *Salmonella* spp. positivity rates of 11.5% in lizards captured on the coast, 3.3% in lizards captured in bushes and 2.5% in lizards captured in the open areas. These researchers reported not to have isolated *Salmonella* spp. from the species *Hoplodactylus chrysosireticus, H. stephensi, H. pacificus, O. moco, C. whitakeri, C. macgregori* and *C. alani*.

In a study by Geue and Löschner (2002) on fecal samples taken from reptile owners and breeders in Germany and Austria, 86 out of 159 samples were found to be culture-positive for *Salmonella* spp., which was a number significantly lower than that detected in snakes and lizards. While only 1 out of 38 fecal samples belonging to tortoises was culture-positive for *Salmonella* spp., *Salmonella* was reported not to have been isolated from the fecal samples of 2 *Uta stansburiana*, 1 *Anolis roquet*, 8 *Eublepharis macularius,* and 1 *Egernia stokesi* lizards. In the present study, *Salmonella* spp. were not identified by culture or the PCR from cloacal swab samples taken from lizards of the species *O. elegans.* Thus, it was determined that *O. elegans* did not serve as a cloacal reservoir for *Salmonella* spp. and in this respect showed similarity to the lizard species investigated in the study by Geue and Löschner (2002).

In a study carried out by Ramos et al. (2019) in Brazil on the presence of *Salmonella* spp. in 76 reptiles, including 15 lizards, 16 tortoises and 45 snakes, *Salmonella* spp., were reported not to have been isolated from 38 out of 45 snakes (84.4%), 7 out of 15 lizards (46.6%) or any of the 16 tortoises. In the present study, *Salmonella* spp. were isolated and identified neither by the PCR technique nor by culture from the cloacal swab samples of 150 snake-eyed lizards of the species *O. elegans*. The comparative assessment of this finding with the results of previous studies suggests that the presence of *Salmonella* spp. in reptiles could be related to the reptilian species.

The increased popularity of keeping reptiles as exotic pet animals (Valdez 2021), these animals not only being kept in terrariums but also being allowed to freely roam in the house, and their continuous contact with their owners pose a public health risk for some variants of *E. coli* and other pathogens belonging to the *Enterobacteriaceae* family. In a study investigating the incidence of *E. coli* in 67 reptiles, including 31 snakes, 18 tortoises and 18 lizards kept as pet animals in Poland, the positivity rate was 16/31 (51.6%) in snakes and 8/18 (44.4%) in lizards and tortoises. While positivity rates for *E. coli* were 83% in *Pogona vitticeps*, 50% in *Iguana iguana*, and 20% in *Furcifer pardali*, *E. coli* was not isolated from *Eublepharis macularius* (Dec et al. 2022). In the present study, the culture analysis of the cloacal swab samples taken from snake-eyed lizards of the species

O. elegans revealed a positivity rate of 16.7% for *E. coli*.

E. coli is a bacterium with genetic heterogeneity and is mostly found as a commensal organism in the intestinal microflora of humans and animals. Some subtypes of this bacterium, which are capable of synthesizing different toxins, cause intestinal or extraintestinal diseases (Gyles 2007). Among these subtypes, enterohemorrhagic *E. coli* (EHEC), and particularly the serotype O157:H7, are known to be definitive human pathogens (Goldwater and Bettelheim 2012). Multiple studies have reported *E. coli* O157:H7 to have been isolated from both animals and humans, and thus have described this strain as having a zoonotic character (Moxley 2004). Apart from being transmitted by animals, *E. coli* O157:H7 is also known as a major foodborne pathogen (Walters et al. 2007, Antaki-Zukoski et al. 2018). In a molecular and culture study conducted by Bautista-Trujillo et al. (2020) on green iguanas kept as pet animals, out of 240 samples 41.7% were found to be positive for *E. coli*, 25.9% were positive for diarrheagenic *E. coli,* 38.7% were positive for Shiga toxin-producing *E. coli,* and 27.4% were positive for enteropathogenic and enteroaggregative *E. coli* pathotypes. Based on these results, pet reptiles were suggested as a major source of infection for human gastrointestinal diseases. The same study reported that the enterohemorrhagic *E. coli* pathotype had not been isolated and identified from any of the samples. In the present study, *E. coli* was isolated and identified from 16.7% of all cloacal swab samples. The positivity rate determined for *E. coli* O157:H7 was 2.7% and the results showed similarity to those previously reported by Antaki-Zukoski et al. (2018). These findings suggest that *O. elegans* is a cloacal carrier of *E. coli* O157:H7 and that the fecal shedding of this serovar by the snake-eyed lizard into the environment could pose a risk to human health.

It is well known that various reptiles, including small lizards, are hunted by many animals and made use of as protein sources. The low oxygen capacity of these reptiles makes it difficult for them to escape from their predators and makes them easy prey (Poulin et al. 2001). *S. enterica* has been reported to have been isolated and identified from 1 out of 100 lizards of the *Agama agama* species captured on a poultry research farm in Nigeria, and it has been suggested that these lizards could transmit *S. enterica* to poultry (Ogunleye et al. 2013). The present study has demonstrated that *Salmonella* spp. may not be found in all reptiles and may be encountered very rarely in some reptilian species.

Conclusion

While previous studies have shown that reptiles may occasionally carry and shed *Salmonella* spp., in the present study, *Salmonella* spp. were not isolated and identified from the snake-eyed lizard *O. elegans*. The results obtained suggest that, while not carrying *Salmonella* spp., *O. elegans* may frequently carry subtypes of *E. coli*, and thereby indirectly affect the health of humans and wild and domestic animals.

To the authors' knowledge, there is no previous study on the microbial flora of the snake-eyed lizard *O. elegans*. Thus, this study could be the first microbiological research on this species and may contribute to future studies in this area.

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