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RESEARCH ARTICLE

Investigation of *Salmonella* in Fresh Water Turtle (*Mauremys caspica caspica,* Gmelin, 1774) from Sewage Discharged Region into Keban Dam Lake, Elazığ [1] [2]

Mikail ÖZCAN * Mustafa SARIEYYÜPOĞLU *

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[2] *This study is summary of master thesis*
* Firat University, Fisheries Faculty, 23119, Elazig - TURKEY

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Summary

In this study, 6 *Salmonella* strains were identified from isolated micro-organisms by culture method in 25 freshwater turtle *(Mauremys caspica caspica)* caught from Koçkale Region of Keban Dam Lake and Haringet strefam (Elazığ City sewage discharged area) in july-october. *Salmonella* isolates was also maked by means of Polymerase Chain Reaction (PCR) technique. According to this result, 6 of 25 turtle were found infected with *Salmonella* spp. *Salmonella* spp. could not be isolated from water samples from study area. In conclusion, *Salmonella* isolated from alive fresh water turtle in Koçkale Region was evaluated that it is threat for human health and fish living in this water.

Keywords: *Fresh water Turtle, Salmonella, PCR, Elazığ, Keban Dam Lake*

Elazığ Şehir Kanalizasyonunun Keban Baraj Gölüne Döküldüğü Bölgeden Yakalanan Tatlı Su Kaplumbağası (*Mauremys caspica caspica,* Gmelin, 1774)'nda *Salmonella*'ların Araştırılması

Özet

Bu çalışmada, Elazığ şehir kanalizasyonunun boşaldığı Haringet çayı ve bu çayın Keban Baraj Gölü'ne döküldüğü Koçkale bölgesinden Temmuz-Ekim 2004 tarihinde yakalanan 25 adet tatlı su kaplumbağası *(Mauremys caspica caspica)*'ndan kültür yöntemi ile izole edilen mikroorganizmalardan 6 suşun *Salmonella* olarak identifikasyonu, Polimeraz Zincir Reaksiyonu (PCR) yöntemi ile teyidi yapılmıştır. Buna göre baraj gölünün Koçkale bölgesinden avlanan 25 kaplumbağadan 6'sının *Salmonella* spp. ile enfekte olduğu tespit edilmiştir. Çalışma sahasından alınan su örneklerinde *Salmonella* spp. varlığına rastlanılmamıştır. Sonuçta, Baraj gölünün Koçkale mevkiinde yakalanan tatlı su kaplumbağasından izole edilen *Salmonella* spp.'nin bu suda yaşayan balık ve insan sağlığı açısından büyük tehdit oluşturduğu ortaya konulmuştur.

Anahtar sözcükler: *Tatlı Su Kaplumbağası, Salmonella, PCR, Elazığ, Keban Baraj Gölü*

INTRODUCTION

Fresh water turtle host to various microorganisms like some other water organisms. *Salmonella* from Enterobacteriaceae family are a gram negative aerobe or facultative anaerobe and rod shaped zoonotic bacteria infected by oral or fecal ways [1]. Salmonellosis is an important problem for both humans and animals [2,3]. *Salmonella* are one of the more important bacteria infecting to humans from turtles directly or indirectly. *Salmonella* which cause of diseases such as typho and paratypho in humans have over 2200 serotypes. So far, 36 serotypes have been isolated from turtles [4,5]. *Salmonella* had been firstly isolated from turtles in 1944, furthermore a direct relationship had been determined between *Salmonella* infections and turtles in 1970-1975. *Salmonella* could also be isolated from many humans directly contacted with reptiles [5].

İletişim (Correspondence)
☎ +90 424 2370000/4567
✉ mikailozcan44@gmail.com

Polymerase chain reaction (PCR) technique has been used for diagnosis of bacterial diseases in medicine and veterinary [6,7]. This technique is more sensitive than traditional culture technique and results in 12-24 h [8-11]. PCR has more advantages than the other techniques for identification of *Salmonella* in terms of specificity and sensitivity [12,13].

In our country there has not been any comprehensive research related to fresh water turtle microbiology. In this study, main aim is the investigation of *Salmonella* with cultivation in fresh water turtle *(Mauremys caspica caspica)* caught from region of Elazığ city sewage discharging into Keban Dam Lake, confirmation of suspicious *Salmonella* by means of PCR and taking attention to effects for human and aquatic animals of environmental pollution agents.

MATERIAL and METHODS

Fresh water turtles were collected from Haringet stream and Koçkale region of Keban Dam Lake in which Elazığ City sewage is discharged between July 2004 and October 2004 Turtle specimens were carried with plastic barrel filled water to Fırat University, Fisheries Faculty, Fish Diseases Laboratory and placed into an aquarium sized 60x40x30cm.

For necropsy, turtles carapace were putted in the middle of cutting board. Ether was applied Turtles for general anesthesia [14]. Afterwards plastron was separated from skin via an electrical angle grinder, gut and liver were brought out into the open as cutting skin and muscles. Whole liver and 1-1.2 g of gut were separately homogenised in a homogenisator with 20% pepton water. Each homogenates were incubated for 24-48 h at 37ºC for preliminary enrichment. 1 ml from this culture was inoculated into rappaport vassiliadis pepton broth and incubated for 24 h at 37ºC. After selective preliminary enrichment, inoculations on Brillant Green Phenol Red Laktoz agar, Xylose Lysine Tergitol 4 agar, MacConcey Agar, Salmonella-Shigella Agar were made aseptically and incubated for 24-48 h at 37ºC. Suspicious *Salmonella* colonies were dyed with Gram stain. Pure colonies were obtained on Nutrient broth and Tryptic soy agar [15-17]. Then, bio-chemical characteristics of these colonies were investigated. H_2S, urease, lysin decarboksilase, ornithin decarboksilase, triptophan deaminase, nitrate reduction, indol, ortho-nitrophenyl-beta-d-galactose (ONPG), lactose, glucose, maltose, mannitol, fermantation tests and oksidase, methyl red, voges proskauer, citrate, catalase were carried out for identification [15,18].

DNA was isolated in order to identify *Salmonella* by means of PCR. For this aim reference *Salmonella* enteritids strain from Fırat University Microbiology Department were used and inoculated into Tryptic Soy agar and specimens were collected from suitable colonies with sterile loop and transferred into tubes containing a volume of 300µl distilled water. Same quantity buffer K (300 µl) and proteinaz K (5 µl) were added to tubes which boiled during 15 minutes after water bath (2 h at 37°C). After boiling, phenol saturated with same quantity (600 µl) Tris-HCl were added and shaken for 5 min properly and centrifugated in 13.000 rpm in a period of 10 min. After this process upper solution in tubes were transferred into another tube by means of micropipet. For presipitation DNA was added 2.5 pure alcohol and 0.1 3M sodium acetate, after suspension was properly mixing, 20ºC 1 h waited, 13.000 rpm 10 h centrifuged and super-natant removed. Precipitate was applied initially a quantity of 300 µl 90% and then 70% alcohol. Between these steps, suspension was centrifugated in 13.000 for 5 min and precipitate obtained were dried for 1 h. After drying, the pellet was made suspension with a quantity of 50-100 µl distilled water and from this suspension were used a volume of 5 µl for PCR.

A volume of 250 µM from each 5 µl 10x PCR buffer, 5 µl 25 mM $MgCl_2$, deoxynucleotides, 50 pmol from 1.25 U Taq DNA Polymerase enzyme, 5 µl template DNA from 16SF1 (5I-TGTTGTGGTTAATAACCGCA-3I) and 16SIII (5I- CACAAATCCATCTCTGGA-3I) primer çiftinin (Promega) 19 primer pairs obtained from *Salmonella* 16S rRNA gene was added into total quantity of 50 µl PCR mixture. PCR reactions were performed in PCR-Sprint Thermocycler (Thermo Hybaid, England). In PCR amplification following the fore denaturation at 94ºC for 2 min, total 35 cycles of PCR as denaturation at 94°C for 45 seconds, as hybridization at 64ºC for 1 min, as synthesis at 72ºC for 2 min were carried out. After last cycle, further synthesis procedure was applied at 72ºC for 10 min.

A volume of 7 µL from DNA products which amplified in PCR were mixed with a quantity of 3 µL blue orange and placed into a cell of agarose gel (1.5%). PCR marker was also placed into last cell. After electrophoresis as using gel, Tris-borik acide-EDTA (TBE) buffer for 1.5 h at 80 V in agarose gel (1.5%) it was dried with volumes of 10 mg/ml and 0.5 µg/ml ethidium bromide for 30 min respectively.

Photographs were taken with a polaroid camera as looking at DNA base pairs peculiar to *Salmonella* in dark room with ultraviolet transilluminator. *Salmonella* enteritidis as a positive control and *Escherichia coli* as a negative control were used both in DNA extracion and in PCR, to detect possible contaminations in any stage of method.

Also fresh water specimens were collected from same regions (5 samples from Haringet and 5 samples from discharged region in Keban dam Lake) and brought to laboratory by sterile plastic bottles. Fresh water specimens were mixed with a volume of 10 ml le pept
order to enrich and 1 ml from this suspension inoculated into Rappaport Vassiliadis Pepton Broth and incubated at 37ºC for 24 h. After selective preliminary enrichment, inoculations onto Brillant Green Phenol Red Laktoz agar, Xylose Lysine Tergitol 4 agar, MacConcey Agar, Salmonella-Shigella Agar were made aseptically and incubated for 24-48 h at 37ºC.

RESULTS

Livers and guts of 25 turtles caught from study fields were examined in term of *Salmonella* and 6 *Salmonella* strain were identified *(Table 1)*.

In these strains, catalase, citrate, ONPG, Lysine decarboxylase, mannitole, nitrate reduction tests for 6 (100%), motility and H_2S tests for 4 (66.6%), glucose test for 3 (50%) and gas creating test for 2 (33.3%) have been detected as positive. On the other hand, oxidase, indole, methyl red, voges proskauer, urease, phenylalanine, tryptophan deaminase and lactose tests for 6 strains (100%), gas creating test for 4 strains (66.6%), glucose for 3 strains (50%) and motility and

Table 1. *Biochemical and morphlological characteristics of 6 Salmonella spp. Isolated from fresh water turtle (Mauremys caspica caspica)*

Tablo 1. *Tatlı su kaplumbağası (Mauremys caspica caspica)'ndan izole edilen 6 Salmonella spp'nin morfolojik ve biyokimyasal özellikleri*

tests	Isolated 6 *Salmonella* spp.					
	1	**2**	**3**	**4**	**5**	**6**
Gram dyed	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-
Catalase	+	+	+	+	+	+
Motility	+	+	-	-	+	+
Hidrogen sulfur (H_2S)	+	-	+	+	-	+
Indole	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-
Voges proskauer	-	-	-	-	-	-
Citrate	+	+	+	+	+	+
Urease	-	-	-	-	-	-
O-Nitrophenyl-β-D-galactopyranosid	+	+	+	+	+	+
Gas creating	+	-	-	+	-	-
Phenylalanine Deaminaz	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	+
Tryptophan deaminase	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+
Lactose Fermantation	-	-	-	-	-	-
Glucose	+	+	-	+	-	-
Mannitole	+	+	+	+	+	+

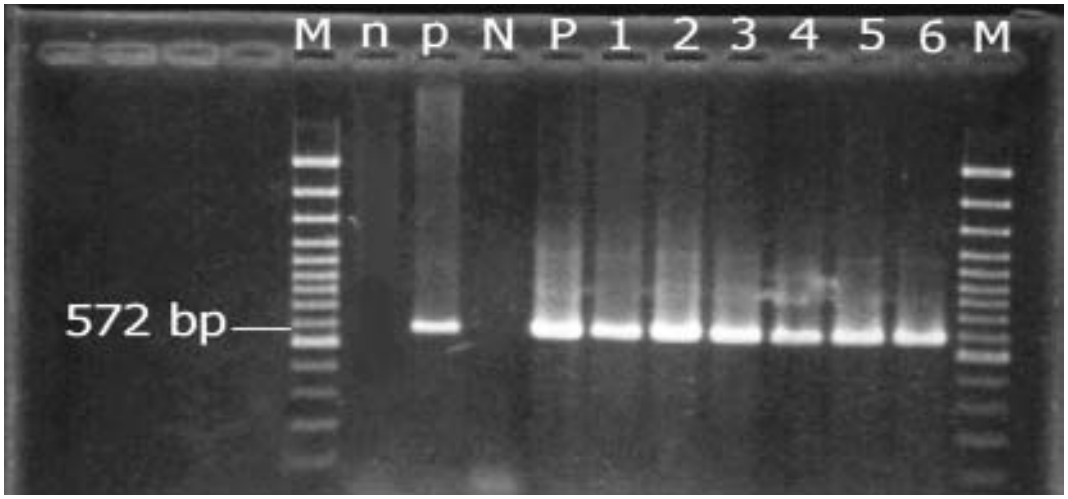

Fig 1. Fresh water turtle *(Mauremys caspica caspica)* isolated DNA *Salmonella* spp., resulted analysis PCR was jel agaroz 1.5% show bands 572 bp coloring with ethidium bromide: Fresh water turtle was obtained sample suspect *Salmonella* spp. M: Marker molecule with 100bp; n: *(Escherichia coli)* (MBI, Fermantas) control negative extraction; p: *(Salmonella enteritidis)* control positive extraction; N: (Steril distile water) control negative PCR; P: (DNA *Salmonella enteritidis*) control pozitive PCR

Şekil 1. Tatlı Su Kaplumbağası *(Mauremys caspica caspica)*'ndan izole edilen *Salmonella* spp. DNA'larının, PCR'nda analizi sonucu oluşan 572 bp'lik bantları gösteren ethidium bromide ile boyanmış % 1.5'luk bir agaroz jel: Tatlı su kaplumbağasından elde edilen *Salmonella* şüpheli örnekler; M: 100 bp'lik moleküler marker; n: Ekstraksiyon negatif kontrol (E. coli) (MBI, Fermantas); p: Ekstraksiyon pozitif kontrol *(Salmonella enteritidis)*; N: PCR negatif kontrol (Steril distile su); P: PCR pozitif kontrol (*Salmonella enteritidis*'in DNA'sı)

H_2S for 2 straind (33.3%) have been detected a negative. According to these results, 6 of these strains had been identified as *Salmonella* spp. *Salmonella* spp. had been isolated in only one (1%) of 10 turtles from Haringet creek and 5 (33.3%) of 15 turtles from Keban Dam Lake. It has been observed that six (24%) of total 25 turtles were infected with *Salmonella* spp. and 19 (76%) were not infected. *Salmonella* spp. was isolated in liver of 2 turtles (8%) and guts of 4 turtles (16%) from 6 infected turtles.

PCR products were applied electrophoresis in agarose gel (1.5%) after amplification with PCR of DNAs taken from suspicious *Salmonella* isolates. Under ultraviolet transilluminator, all the samples (6 samples) were 572 bp in length and were the same with positive control. After these outcomes, 6 suspicious strains were proved as *Salmonella* spp. *(Fig 1)*.

In the water samples from study field, a lot of *E. coli* strains were identified, but *Salmonella* spp. strain was not come across.

DISCUSSION

In this study, Isolation and identification of *Salmonella* with cultivation method have been carried out in fresh water turtle caught from Haringet creek and from a region of Keban Dam Lake in which sewage of Elazığ city has been discharged. Suspected samples in term of *Salmonella* have been successfully identified by using PCR technique.

Many researches have informed that domestic and industrial wastes with microorganisms have especially polluted to discharged regions and environment. Salmonella is also one of the most important bacteria because of their infectivity [20-24]. In previous study [25] Coliform bacteria, especially *E. coli* had been detected in both water and mussels abundantly in Koçkale region of Keban Dam Lake and it has been pointed out that these regions contain too much polluting agents microbiologically. In the present study a lot of *E. coli* has included in the water samples collected from the same regions and this finding has proved one more time to be more pollution of this region.

In traditional identification of *Salmonella*, bio-chemical and serological tests have been used widespread. But these classical techniques may give some wrong results especially in state of impure strains. It has been expressed that *Salmonella* growth rapidly, even though they need about 7-8 days for isolation and identification [28]. However bacteria within Enterobacteriaceae have been isolated easily as using Lassen triple tube system [15,16,25-27]. In this study, Lassen triple tube systems have been successfully applied on turtles. Becuase it given results in a short time like 3 days biochemically.

In previously studies [25,29] Salmonella was isolated in guts of fish from Koçkale region. Şeker et al.[31] isolated *Salmonella* spp. in mussels *(Unio elongatulus eucirrus)* from the same region. All these studies have shown that the contamination of aquathic organims with *Salmonella* have been continuing in this region of Keban Dam Lake.

Pasman et al.[32] contaminated with *Salmonella* to 5 turtles *(Trachemys scripta scripta)* 5x105 dose orally and to the other 5 turtles 5x105 dose intraperitoneally.

This two group turtles were kept at 26ºC and 37ºC respectively and blood, liver, spleen, gut specimens from turtles died after 35th days were investigated using ELISA. *Salmonella* was detected in blood, liver, spleen and gut samples. Pasman et al.[33] investigated *Salmonella* in guts of turtles using electron microscopy and perceived that *Salmonella* adhered to gut mucosa and epithelium. In the present study *Salmonella* was isolated from livers of 2 (8%) turtles and guts of 4 (16%) turtles. This finding has also been supported by former researcher's findings. It has been described that PCR is a more advantages technique than traditional and serological tests [19] because of it is accuracy and to be easy for lower quantity of strains and pure or mixed strains to identify in a short time periods like 1-2 days. Despite of PCR is not direct identification technique, using specific primers after ampification gets easy to recognize bacteria. In our study wrong positive reactions of different bacteria were obstructed using genus specific *Salmonella* 16S FI rRNA gene and *Salmonella* 16S FII rRNA gene primers. PCR were performed with negative control and there were no contaminations in any stages.

In a former study [31] *Salmonella* did not isolate from water samples in Koçkale region. In the present study, *Salmonella* strains were also not detected in water samples from Haringet creek and Keban Dam Lake.

In conclusion, It has been determined that turtles *(Mauremys caspica caspica)* caught from Koçkale Region of Keban Dam Lake and Haringet stream (Elazığ City sewage discharged area) have carried *Salmonella* causing serious infections with contaminations of

environment and are pathogens for human and the other organisms. Because turtles that is carrying *Salmonella* showed no clinical signs. It was considered that this organism only carries *Salmonella* agents. If biological systems are saved from polluting effects, continuousness may be provided in these systems. This study is important because *Salmonella* spp. was found in turtles which belong to aquatic systems. It has showed that the continuance of *Salmonella* spp. may possibly cause bigger problems in the future, so precautions must be taken for preventing of this problem.

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