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RESEARCH ARTICLE **ARAŞTIRMA MAKALESİ**

Bacterial agents isolated from cultured marsh frog (*Pelophylax ridibundus*, Pallas 1771)

Yetiştiriciliği yapılan ova kurbağasından (*Pelophylax ridibundus*, Pallas 1771) izole edilen bakteriyel etkenler

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Abstract: Marsh frogs (*Pelophylax ridibundus*) are preferred in European cuisine. In recent years, interest in farming of marsh frogs has increased, but little is known about their bacterial diseases. This research was carried out in a marsh frog farming operation in Mersin, Turkey, in order to determine the bacterial diversity. For this purpose, a total of 339 frog, 30 water, and 8 feed samples were collected. Isolation and identification of bacteria were carried out by conventional techniques and the VITEK-2 compact system. Antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion method. A total of 239 isolates of 49 different species, including 31 Gram negative rod-shaped bacteria, 9 Gram positive rod-shaped sporeforming bacteria, and 9 Gram positive cocci-shaped non-sporeforming bacteria have been identified. These bacteria species were detected from 25 (83.3%) water, 5 (62.5%) feed samples, and 64 (84.2%) of 76 frog specimens. Antimicrobial susceptibility and MAR index values ranged between 1.4-95.8% and 0.13-0.73, respectively. In conclusion, the presence of opportunistic pathogenic bacteria in water, feed and frog specimens, which could pose risk for frogs and human health, have been detected in the marsh frog farm in Mersin. This study reveals, that further investigations are necessary for sustainable marsh frog breeding in Turkey.

Keywords: *Bacteria, frog disease, raniculture, marsh frog, Pelophylax ridibundus*

Öz: Ova kurbağası (*Pelophylax ridibundus*) Avrupa mutfağında tercih edilen bir türdür. Son yıllarda kurbağa yetiştiriciliğine ilgi artmış olmasına karşın bakteriyel hastalıkları hakkında çok az şey bilinmektedir. Bu araştırma, bakteri çeşitliliğini tespit etmek amacıyla Mersin'de bir ova kurbağası çiftliğinde gerçekleştirilmiştir. Bu amaçla toplam 339 kurbağa, 30 su ve 8 yem örneği toplanmıştır. Bakterilerin izolasyonu ve tanımlanması geleneksel teknikler ve VITEK-2 kompakt sistemi ile gerçekleştirilmiştir. Antimikrobiyal duyarlılık testi Kirby-Bauer disk difüzyon yöntemi ile yapılmıştır. 31 Gram negatif basil, 9 Gram pozitif basil ve 9 Gram pozitif kok içeren 49 farklı türden toplam 239 izolat tanımlanmıştır. Bu bakteri türlerinin 25'i (% 83,3) su, 5'i (% 62,5) yem örneklerinden ve 64'ü (% 84,2) 76 kurbağa numunesinden tespit edilmiştir. Antimikrobiyal duyarlılık ve MAR indeksi değerleri sırasıyla % 1,4-95,8 ve 0,13-0,73 arasında değişmektedir. Sonuç olarak, Mersin'deki ova kurbağası çiftliğinde kurbağa ve insan sağlığı açısından risk oluşturabilecek fırsatçı patojenik bakterilerin varlığı kurbağa, su ve yem örneklerinde tespit edilmiştir. Bu çalışma, Türkiye'de sürdürülebilir ova kurbağalarının yetiştirilmesi için daha ileri araştırmaların gerekli olduğunu ortaya koymaktadır.

Anahtar kelimeler: *Bakteri, kurbağa hastalığı, kurbağa yetiştiriciliği, ova kurbağası, Pelophylax ridibundus*

INTRODUCTION

Frogs, one of the delicacies of the world cuisine, are also used as pets, education and research tools, and different industries (Pasteris et al., 2006). Frogs caught from the nature till the beginning of the 20th century are now successfully cultivated in South America and some Far Eastern countries, especially the American bull frog (*Lithobates catesbeianus*, Shaw 1802) (Amborski et al., 1983). Some frog species of our country's natural habitat are an important export item for Europe. Since production through cultivation has become mandatory, because of increased demand and reduced natural resources, extensive and semi-intensive farming of marsh frog (*Pelophylax ridibundus*) has been conducted by some private and public institutions in recent years. However, intensive raniculture is still in

experimental status in Turkey. Unfortunately, farming operations often lead to increased risk of diseases and mortality (Pasteris et al., 2006). Within the aquatic environment, frogs are in contact with a number of potentially pathogenic bacteria. Stressed conditions like crowding or unsanitary lead to overcome weakened immune barriers and cause disease (Mauel et al., 2002). Causative agents of bacterial diseases in many frog species, both wild (Schadich and Cole, 2010) and cultivated (Amborski et al., 1983; Mauel et al., 2002; Huys et al., 2003; Pasteris et al., 2006; Pasteris et al., 2009; Pasteris et al., 2011; Pilarski & Schocken-Iturrino, 2011; Jeong et al., 2014; Xiaoying et al., 2015), and in water samples (Pasteris et al., 2006; Hacioglu et al., 2015) have been reported previously. However, as far as literature reviews are concerned, no reports about bacterial agents of marsh frogs have been found. According to our knowledge, this is the first research about bacteria in cultured marsh frogs.

To detect the efficacy of drugs, antimicrobial susceptibility testing has been conducted. Different rates of antimicrobial susceptibility tests of bacterial isolates from various frog species were reported (Lee et al., 2009; Pilarski and Schocken-Iturrino, 2011; Tee and Najiah, 2011; Hacioglu et al., 2015). In order to indicate the intensity of exposure of antibacterial drugs to bacteria, some investigators have reported multiple antibiotic resistance (MAR) index values of isolates from reared American bull frogs (Lee et al., 2009; Tee and Najiah, 2011) and some wild frog species (Hacioglu and Tosunoglu, 2014; Hacioglu et al., 2015).

There is a great interest in frog farming in Turkey and a potential for development of frog farming due to expanding demand in export markets. Therefore, the aim of this study was to shed light on the emerging diseases of farmed marsh frogs, which possess a promising development potential in our country.

MATERIALS AND METHODS

Frog, water and feed samples

Samples were collected from the pilot-scale marsh frog farm in Aydıncık, Mersin, Turkey. For ungoing of the farm, full-cycle production and hatchery-rared system, and unchlorinated underground-water was used. Water temperatures ranged between 16ºC and 28ºC during the sampling dates. Frogs were fed with ground and pellet feed.

A total of 339 frog (Table 1), 30 water (8 tap and 22 pool water), and 8 pellet feed (7 farm-made frog feed and one commercial trout feed) samples were collected randomly 8 times according to metamorphic stages between 30.03.-12.10.2017. All samples were brought under adequate conditions to the laboratory and processed on same day. Whereas water and pellet feed samples were transported in aseptic and cooled conditions, all frog samples were carried alive in water included plastic containers. Life frog samples were examined for external findings; weight and length measurements before any treatment (Table 1).

Microbiological analysis

After euthanasia of frog samples by transdermal exposure of buffered MS-222 (% 1) (Hacioglu et al., 2014) and disinfection by povidin/iodin solution (400 ppm) (Brown et al., 1997), dissection has been applicated (Whitaker & Wright, 2001) and clinical signs have been recorded.

Bacteriological analysis of frog specimens were carried out on whole eggs, embryos (4 days old), and larvae; heads and bodies without internal organs of tadpoles and baby frogs; and lungs, liver, spleen, blood and lesions of juvenile frogs and adult frogs. Since similar samples were combined and homogenized, a total of 76 frog specimens of 339 samples were studied (Table 1). Isolation of bacteria have been made by conventional methods (Austin & Austin, 2007). Frog specimens were streaked directly onto Trypticase Soy Agar (TSA; Merck) and Tryptone Yeast Extract Salts Agar (TYESA) (Brown et al., 1997). For water and feed samples 0.3 mL of appropriate three-fold serial dilutions, prepared with peptone water (PW) have been used. Incubation time for TSA was 48-72 hours at 30°C and 5-7 days at 15°C for TYESA. Different colonies on TSA and yellow colonies on TYESA have been subcultured and used for identification. Isolates were stored at -20°C in Nutrient broth or TYES Broth supplemented with 15% (v/v) glycerol. Bacteria were also indentified by the VITEK-2 compact system (bioMerieux, France) (VITEK-2 GN for Gram negative rod-shaped bacteri, VITEK-2 GP for Gram positive cocci-shaped bacteria and VITEK-2 BCL ID cards for Gram positive rod-shaped bacteria) according to the manufacturer's instructions. Before carrying out the VITEK tests, some traditional identification tests such as Gram, catalase, cytochrome oxidase, motility and flexirubin (Plumb and Browser, 1983) have been performed.

Antimicrobial susceptibility testing and multiple antibiotic resistance (MAR) index

Antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion method according to the Clinical and Laboratory Standart Institute (CLSI, 2010). The incubation was carried out at 30°C for 24-48 hours. For this purpose, amoxycillin (AMX-10 µg), clindamycin (CC-2 µg), ofloxacin (OFX-30 µg), penicillin (P-10 µg) (BD BBL, USA), doxycycline (D-30 µg), enrofloxacin (ENR-5 µg), eritromycin (E-15 µg), florfenicol (FFC-30 µg),

Table 1. Weight and length of frog samples according to metamorphic phases

					Weight (g)		Length (cm)			
	Metamorphic				Min.-Max.	Mean±SD	Min.-Max.		Mean ±SD	
Date	Stages	n	ns	nsb			NS	NL/NT	NS	NL/NT
30.03.17	Adult frog	10	10	7	18.23-46.37	37.77±7.91	6.0-8.8	15.0-18.5	7.18±0.60	16.67±1.0
	Egg	26	1	1	NM	NM		NM		0.16
	Embryo	53	3	3	NM	NM		0.2-0.45		0.32±0.08
06.04.17	YSL-4	51	2	2	NM	NM		0.7-1.0		0.85±0.09
	YSL-8	41	2	2	NM	NM		0.9-1.0		0.97±0.05
20.04.17	Fed larvae	20	2	2	0.01-0.08	0.03±0.02		1.0-1.7		1.24±0.22
	Diseased larvae	20	2	2	NM	NM		0.6-1.6		1.02±0.24
12.06.17	Tadpole	18	2	2	0.17-0.35	0.27±0.06		1.5-4.1		2.59±0.66
30.06.17	Tadpole	13	1	1	0.34-0.90	0.53±0.15		3.5-4.7		4.09±0.39
30.06-12.07.17	T hindlimb	13	2	2	0.99-2.21	1.5±0.4		4.8-6.5		5.57±0.6
30.06.17	T four limbs	3	1	1	1.75-1.9	1.82±0.08		6.2-6.5		6.33±0.15
12.07.17		4	2	2	0.53–1.66	1.09±0.33		3.5–5.5		4.55±0.82
10.08.17		12	2	2	0.57-1.68	1.02±0.3		3.0-5.0		4.34±0.62
12.07.17	Baby frog	9	2	2	0.39-0.92	0.67±0.19	1.5-2.0	3.5-5.0	1.8±0.2	4.37±0.52
12.07.17	Diseased tadpole	6	1	1	0.66-2.60	1.64±0.89		4.0-6.5		4.88±1.18
10.08.17	Baby frog	23	23	18	0.56-2.4	1.14±0.53	1.5-3.0	3.0-7.0	2.1±0.34	4.78±1.02
12.10.17	Juvenil frog	14	14	10	4.46-40.20	16.75±9.16	3.5-7.5	9.0-16.5	5.33±0.96	12.8±2.09
	Diseased adult frog	4	4	4	24.08-39.0	33.66±6.9	6.2-7.5	15.5-18.0	6.92±0.53	17.0±1.22
	Total	**339**	**76**	**64**						

n: Number of samples, ns: Number of specimens, nsb: Number of bacteria detected specimens, NM: Not measured, NS: Nose-sacrum, NL: Nose-limb, NT: Nose-tail, YSL-4: Yolk-sac larvae, 4 days old, YSL-8: Yolk-sac larvae, 8 days old.

gentamicin (GM-10 µg), lincomycin (L-2 µg), neomycin (N-30 µg), oxytetracycline (T-30 µg), streptomycin (S-10 µg), trimethoprim-sulfamethoxazole (SXT-23,75-1,25 µg) and vankomycin (Va-30 µg) (Bioanalyse, Turkey) discs were used.

MAR index of the isolates were calculated according to Krumperman's method (Lee et al., 2009) as follows: [*MAR index = X / (Y x Z)*]

X: Total count of bacteria resistant to antibacterial drugs, Y: Total antibacterial drug count used,

Z: Total isolate count

While MAR index values higher than 0.20 indicate, that the animal was exposed to antibacterial drugs, values equal to or less than 0.20 suggests, that drugs were rarely or never used for therapeutic purposes (Lee et al., 2009). *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 25923) were used as reference strains.

RESULTS

External and internal symptoms have been rarely observed in frog samples. Air bubbles were detected in the abdomen of 14-21 day old larvae. At this phase, the mortality rate has exceeded 80%. External symptomes like swollen abdomen and gas bubble at the vent in tadpoles, spinal deformities, ulcerative lesions, swelling and weakness in limb muscles in

tadpoles and adult frogs were observed. Internal signs like haemorrhages and ulcerative injuries in muscles, anemia and haemorrhages in intestines and liver have been detected in adult frogs (Figure 1).

In all examined samples, a total of 239 isolates of 49 different species were identified, of which 31 (63.26%) were Gram negative rod-shaped bacteria, 9 (18.37%) were Gram positive rod-shaped sporeforming bacteria, and 9 (18.37%) were Gram positive cocci-shaped non-sporeforming bacteria. Of these isolates, 172 (72%) have been detected from 64 (84.2%) of 76 frog specimens, 60 (25.1%) from 25 (83.3%) of 30 water, and 7 (2.9%) from 5 (62.5%) of 8 feed samples. According to literature, some detected bacteria of this study are reported for the first time in frogs (Tables 2, 3, and 4).

Antimicrobial susceptibility testing was performed on 71 bacterial isolates of the 239 bacterial isolates, covering 31 different species. Antimicrobial susceptibility ratios ranged between 1.4 and 95.8% (lincomisin and enrofloxacin) (Table 5).

MAR index values of 26 of 31 isolates (83.87%) have been detected higher than 0.20. Resistance, susceptibility and MAR index values of the isolates can be seen on Table 6.

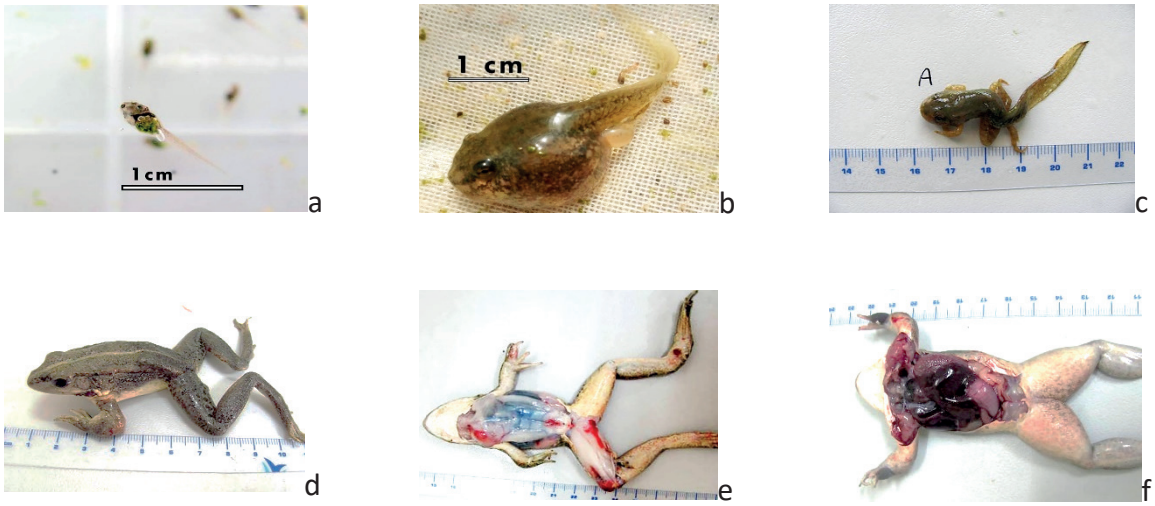

Figure 1. External and internal pathological features of marsh frog specimens

a. Fourteeen days old swimming larvae, air bubbles in abdomen

b. Tadpoles with swollen abdomen and gas bubble at the vent

c. Scoliosis in tadpole, four limbs stage

d. Spinal deformities, ulcerative lesions, swollen and weak muscles of limbs, adult stage

e. Haemorrhages and ulcerative lesions in muscles, adult stage

f. Haemorrhages in intestines and liver, adult stage

Table 2. Number of the Gram negative rod-shaped bacteria according to samples

No	Gram negative rod-shaped bacteria	Frog	Water	Feed	Total
1	*Acinetobacter haemolyticus*	3	0	0	**3**
2	*Aeromonas hydrophila/ caviae*	12	7	0	**19**
3	*Aeromonas sobria*	12	4	0	**16**
4	*Bordetella hinzii*	0	1	0	**1**
5	*Brevundimonas diminuta/ vesicularis*†	6	0	0	**6**
6	*Chryseobacterium indologenes*	2	5	0	**7**
7	*Citrobacter braakii*	0	1	0	**1**
8	*Citrobacter freundii*	26	2	0	**28**
9	*Delftia acidovorans*†	3	1	0	**4**
10	*Edwardsiella tarda*	18	2	0	**20**
11	*Elizabethkingia meningoseptica*	1	0	0	**1**
12	*Enterobacter asburiae*	0	3	0	**3**
13	*Escherichia coli*	7	0	0	**7**
14	*Klebsiella oxytoca*	1	0	0	**1**
15	*Klebsiella pneumoniae ozaenae*	1	0	1	**2**
16	*Morganella morganii*†	2	1	0	**3**
17	*Pantoea* spp.	0	1	1	**2**
18	*Plesiomonas shigelloides*	1	0	0	**1**
19	*Providencia rettgeri*†	0	1	0	**1**
20	*Pseudomonas aeruginosa*	1	2	0	**3**
21	*Pseudomonas fluorescens*	0	2	0	**2**
22	*Pseudomonas mendocina*†	0	1	0	**1**
23	*Pseudomonas putida*	1	1	0	**2**
24	*Rhizobium radiobacter*†	0	1	0	**1**
25	*Salmonella* spp.	13	1	0	**14**
26	*Serratia fonticola*†	0	1	0	**1**
27	*Serratia odorifera*†	0	1	0	**1**
28	*Shewanella putrefaciens*	11	1	0	**12**
29	*Sphingomonas paucimobilis*†	10	5	0	**15**
30	*Sphingobacterium t halpophilum*	0	1	0	**1**
31	*Stenotrophomonas maltophilia*†	0	1	0	**1**
	Total	**130**	**47**	**2**	**179**

†First isolation in frogs

Table 3. Number of the Gram positive rod-shaped sporeforming bacteria according to samples

No	Gram positive rod-shaped sporeforming bacteria	Frog	Water	Feed	Total
1	*Bacillus cereus/mycoides/ thuringiensis*	9	5	2	**16**
2	*Bacillus clausii*	1	0	1	**2**
3	*Bacillus fortis*	1	0	0	**1**
4	*Bacillus megaterium*	1	1	0	**2**
5	*Bacillus pumilus*	1	0	0	**1**
6	*Bacillus smithii*	0	1	2	**3**
7	*B.subtilis/amyloliquefaciens/atropha*†	2	2	0	**4**
8	*Brevibacillus choshinensis*	1	0	0	**1**
9	*Lysinibacillus sphaericus/ fuciformis*†	1	0	0	**1**
	Total	**17**	**9**	**5**	**31**

†First isolation in frogs

Table 4. Number of the Gram positive cocci-shaped non-sporeforming bacteria according to samples

No	Gram positive cocci-shaped non-sporeforming bacteria	Frog	Water	Feed	Total
1	*Aerococcus viridans*†	1	1	0	**2**
2	*Enterococcus gallinarum*†	2	0	0	**2**
3	*Granulicatella adiacens*†	1	0	0	**1**
4	*Kocuria kristinae*†	0	1	0	**1**
5	*Kocuria rhizophila*†	2	1	0	**3**
6	*Kocuria rosea*†	1	0	0	**1**
7	*Micrococcus luteus/lylae*†	16	0	0	**16**
8	*Staphylococcus aureus*	0	1	0	**1**
9	*Staphylococcus equorum*†	2	0	0	**2**
	Total	**25**	**4**	**0**	**29**

†First isolation in frogs

DISCUSSION

Although there were no serious clinical signs in adult frogs, high mortalities continued in 2-3 weeks old larvae from April to July 2017 and the final mortality ratio exceeded 80% during the present study. Whereas D'Silva (2015) reported an economically tolerable mortality rate in frog growth of 20% in the spawning phase, 10% in tadpole phase, 35% in baby and juvenile phase and 10% in the fattening phase. We estimate that the high mortality rate of larvae was due to gas bubble disease, because massive greening of water

Table 5. Susceptibility and resistance rates of antimicrobial drugs of the isolates

	Gr- rod-shaped bacteria		Gr+ rod-shaped bacteria		Gr+ cocci-shaped bacteria		Total	
Antimicrobial drugs	S %	R %	S %	R %	S %	R %	S %	R %
Lincomycin	0	100	6.7	93.3	0	100	1.4	98.6
Penicillin	2.2	97.8	26.7	66.7	50.0	41.7	15.3	81.9
Clindamycin	4.4	91.1	40.0	53.3	41.7	41.7	18.1	75.0
Amoxicillin	24.4	75.6	40.0	60	66.7	33.3	34.7	65.3
Vancomycin	6.7	91.1	86.7	6.7	33.3	33.3	27.8	63.9
Erythromycin	6.7	68.9	26.7	13.4	33.3	33.3	13.9	50.0
Neomycin	35.6	31.1	80.0	0	33.3	41.7	44.4	26.4
Streptomycin	44.4	31.1	73.3	20.0	58.3	8.3	52.8	25.0
SXT	75.6	22.2	66.7	33.3	83.3	16.7	75.0	23.6
Oxytetracycline	31.1	33.3	73.3	6.7	66.7	8.3	45.8	23.6
Florfenicol	68.9	26.7	93.3	6.7	75.0	16.7	75.0	20.8
Gentamycin	73.3	20.0	93.3	0	66.7	16.7	77.1	14.3
Ofloxacin	93.3	4.4	80.0	0	75.0	8.3	87.5	4.2
Doxycycline	86.7	6.6	93.3	0	100	0	90.3	4.2
Enrofloxacin	97.8	0	100	0	83.3	8.3	95.8	1.4

S: susceptibility, R: resistance, SXT: Trimethoprim-sulfamethoxazole

and, especially in the afternoons, increased air bubbles on the water surface of pools and in the abdomen of tadpoles were observed (Figure 1a). Indeed, Lutz and Avery (1999) reported that tadpoles are particularly susceptible to gas bubble disease caused by the oxygen supersaturation associated with afternoon algal photosynthesis.

The most common disease of frogs is the red-leg syndrome, also known as bacterial dermatosepticemia. External symptomes like anorexia, lethargy, discolorations, hemorrhages, ulcers, necrosis, swelling due to subcutaneous edema, focal hemorrhages in skin and skeletal muscles, especially of the limbs; assites, discoloration, and megali of liver and spleen, and other pathological findings such as hemorrhages in internal organs have been reported (Taylor et al., 2001; Pasteris et al., 2006). Although some of the symptoms detected in this study may indicate dermatosepticemia; it can be suggested that these findings could be accidental, because they were present only in a few adult frogs and point to other diseases as well.

Most of the 61 isolates of water samples were *Aeromonas hydrophila/caviae*, *Chryseobacterium indologenes*, *Sphingomonas paucimobilis*, *Bacillus cereus/mycoides/thuringiensis*, *Aeromonas sobria*, *Enterobacter asburiae* and *Citrobacter freundii,* respectively (Table 2, Table 3). Hacioglu et al. (2015) detected in water samples from the environment of wild frogs (including *P. ridibundus*) *A. hydrophila/caviae*, *Pseudomonas aeruginosa*, *Ps. fluorescens* and *Shewanella putrefaciens* similar; and *Actinobasillus* sp., *Elizabethkingia meningoseptica*, *Enterobacter gergoviae*, *Escherichia coli, Klebsiella oxytoca*, *Moroxella* sp., *Pasteurella multocida, Serratia liquefasciens*, *Ser. rubidaea* and *Vibrio carchariae* dissimilar compared to our water findings. Aeromonads, natural members of the aquatic environment (Austin and Austin, 2007), have been reported in previous studies in various diseases of frogs, especially in cases of red-leg sydrome (Mauel et al., 2002; Huys et al., 2003; Pasteris et al., 2006; Lee et al., 2009).

Previously, *Citrobacter freundii* was reported in gut microbiota of American bull frogs (Miles et al., 2004), internal organs of diseased *Rana dybowskii* (Jeong et al., 2014) and as agent of the red-leg sydrome (Pasteris et al., 2011). In this study, the presence of *Citrobacter freundii* in both frog and water samples indicates, that it can be an opportunistic pathogen for *P. ridibundus*. *Edwardsiella tarda,* the etiological agent of edwardsiellosis of catfish and eel (Austin and Austin, 2007), was detected in both frog and water samples of four-limbed tadpoles, baby and juvenile marsh frog pools. *Klebsiella pneumoniae ozaenae*, isolated in both tadpoles and feed in this study, has been reported

Table 6. Resistance, susceptibility and MAR index values of the isolates

No	Gram negative rod-shaped bacteria	n	R	I	S	R %	S %	MAR
1	*Acinetobacter haemolyticus*	1	8	1	6	53.3	40.0	0.53
2	*Aeromonas* spp.	10	67	15	68	44.7	45.3	0.45
3	*Bordetella hinzii*	1	6	1	8	40.0	53.3	0.40
4	*Chryseobacterium indologenes*	1	12	0	3	80.0	20.0	0.80
5	*Citrobacter* spp.	2	13	4	13	43.3	43.3	0.43
6	*Delftia acidovorans*	1	6	2	7	40.0	46.7	0.40
7	*Edwardsiella tarda*	1	5	1	9	33.3	60.0	0.33
8	*Elizabethkingia meningoseptica*	1	8	2	5	53.3	33.3	0.53
9	*Enterobacter asburiae*	1	6	1	8	40.0	53.3	0.40
10	*Escherichia coli*	2	12	1	17	40.0	56.7	0.40
11	*Klebsiella* spp.	2	8	3	19	26.7	63.3	0.27
12	*Morganella morganii*	1	5	2	8	33.3	53.3	0.33
13	*Pantoea* spp.	2	8	5	17	26.7	56.7	0.27
14	*Plesiomonas shigelloides*	1	7	2	6	46.7	40.0	0.47
15	*Providencia rettgeri*	1	8	1	6	53.3	40.0	0.53
16	*Pseudomonas* spp.	6	55	7	28	61.1	31.1	0.61
17	*Salmonella* spp.	5	36	11	28	48.0	37.3	0.48
18	*Serratia* spp.	2	13	2	15	43.3	50.0	0.43
19	*Shewanella putrefaciens*	1	5	1	9	33.3	60.0	0.33
20	*Sphingomonas paucimobilis*	1	8	3	4	53.3	26.7	0.53
21	*Sphingobacterium thalpophilum*	1	8	2	5	53.3	33.3	0.53
22	*Stenotrophomonas maltophilia*	1	11	0	4	73.3	26.7	0.73
	Total	**45**	**315**	**67**	**293**	**46.7**	**43.4**	**0.47**
	Escherichia coli (ATCC25922)	1	6	0	9	40	60	0.40
	Gram positive rod-shaped sporeforming bacteria							
23	*Bacillus spp.*	13	49	23	123	25.1	63.1	0.25
24	*Brevibacillus choshinensis*	1	2	1	12	13.3	80.0	0.13
25	*Lysinibacillus sphaericus/fuciformis*	1	3	0	12	20.0	80.0	0.20
	Total	15	54	24	147	24	65.3	0.24
	Gram positive cocci-shaped non-sporeforming bacteria							
26	*Aerococcus viridans*	1	3	2	10	20.0	66.7	0.20
27	*Enterococcus gallinarum*	1	3	5	7	20.0	46.7	0.20
28	*Granulicatella adiacens*	1	7	1	7	46.7	46.7	0.47
29	*Kocuria spp.*	3	18	10	17	40.0	37.8	0.40
30	*Micrococcus luteus/lylae*	4	9	7	44	15.0	73.3	0.15
31	*Staphylococcus spp.*	2	8	4	18	26.7	60.0	0.27
	Total	12	48	29	103	26.7	57.2	0.27
	Enterococcus faecalis (ATCC29212)	1	5	2	8	33.3	53.3	0.33
	Staphylococcus aureus (ATCC25923)	1	1	1	13	6.6	86.6	0.06

n: number of isolates, S: susceptibility, R: resistance, I: intermediate

to cause haemorrhage, ulcer and reddening of the abdomen in brown tree frogs (*Litoria ewingii*) (Mauel et al., 2002). As a matter of fact, these coliforms are widespread in nature, microbiota and opportunistic pathogens of humans and animals (Holt et al., 2000).

The most dominant species isolated in liver, lung and / or blood specimens of juvenile and adult frog specimens were *Salmonella* spp. They have also been detected in pool water of juvenile frogs. It is remarkable that *Salmonella* spp. appeared only in adult and juvenile frogs. Salmonellae, which have only two species and more than 2500 serotypes (LPSN, 2017), are mostly pathogenic to human and cause severe infections ranging from simple gastrointestinal disorders to death (Holt et al., 2000). Although common in nature, intestines of humans, warm-blooded and cold-blooded animals, *Salmonella* spp. are known nonpathogenic for aquatic animals (CFSPH, 2013). In fish disease analysis, bacteria can be detected in internal organs only in presence of an infection. Therefore, the relationship of *Salmonella* and frogs should be investigated, because of their presence in liver and lungs. Since only the internal organs of adult frogs were examined in this study, it would be appropriate to investigate also the meat of frogs for *Salmonella* spp. and other potentially pathogenic bacteria.

Acinetobacter haemolyticus, *A. hydrophila / caviae* (Miles et al., 2004; Pasteris et al., 2006), *C. freundii*, *E. coli*, *Klebsiella* sp., *Plesiomonas shigelloides*, *Pseudomonas aeruginosa*, *Ps. putida, Bacillus cereus / mycoides / thuringiensis* and *Micrococcus* sp. (Miles et al., 2004), reported from the intestines of the healthy American bull frogs, were also detected in this research. The findings of *A. hydrophila / caviae*, *C. freundii* (Hacioglu and Tosunoglu, 2014; Hacioglu et al., 2015), *E. coli*, *K. pneumoniae ozaenae* (Hacioglu and Tosunoglu, 2014) and *Salmonella* spp. (Hacioglu et al., 2015), isolated by mouth and cloacal swaps of wild frogs in our country (Hacioglu and Tosunoglu, 2014; Hacioglu et al., 2015), were identical to ours. Taylor et al. (2001) reported that *Enterobacter* sp., *E. coli*, *K. ozaenae*, *Plesiomonas shigelloides*, *Salmonella* spp., *B. cereus / mycoides / thuringiensis*, *B. megaterium*, *Micrococcus* sp. and *Staphylococcus* spp., also present in our findings, are usually nonpathogenic to amphibians due to their presence in their normal microbiota. Nevertheless, it has been emphasized that these bacteria should be considered as causative agents, if detected in blood or coelomic cavity or at very high rates (Taylor et al., 2001). Thus, *Acinetobacter* sp. (Jeong et al., 2014), *A. hydrophila/caviae* (Mauel et al., 2002; Huys et al., 2003), *Aeromonas* sp. (Amborski et al., 1983), *Chryseobacterium indologenes*, *Citrobacter braakii*, *E. tarda* (Tee and Najiah, 2011), *Enterobacter* sp., *E. coli* (Pasteris et al., 2009) and *Pseudomonas* sp. (Amborski et al., 1983; Tee and Najiah, 2011) were reported in diseased frogs. In contrast, Schadich and Cole (2010) found that *A. hydrophila/ caviae* was harmless to *Litoria ewingii*. Although lack of serious findings of red-leg syndrome, previous reported etiologic agents like *A. hydrophila* (Pilarski and Schocken-Iturrino, 2010; Pasteris et al., 2011; Tee and Najiah, 2011; Jeong et al., 2014; Xiaoying et al., 2015), *E. tarda*, *Chryseobacterium indolgenes*, *Pseudomonas* spp. (Tee and Najiah, 2011) and *K. pneumoniae* (Schadich and Cole, 2010) were also identified in this study. It was reported that *Staphylococcus* species, found in the pool water of tadpoles (*S. aureus*) and juvenile frogs (*S. equorum*) in the present study, cause general edema and whirling diseases in farmed bullfrogs (FAO, 2016). Nevertheless, the pathogenic effects of these bacteria on marsh frogs should be investigated. It is obvious, that most of the isolates of this investigation are natural microbiota of water and frog specimens. However, it should be taken into consideration, that many bacteria seize opportunities in stress situations. For this reason, the pathogenicity and virulence of these isolates should be determined in cultivated frog species.

Despite resistance problems, antibacterial drugs are still used extensively in the treatment of bacterial diseases. Lee et al. (2009), detected resistance to lincomycin (90-95%), amoxicillin (72.5-80%), oxytetracycline (70-75%), erythromycin (65-75%), sulfamethoxazole (47.5-42.5%), doxycycline (47.5-50%) and florfenicol (10-0%) against *Aeromonas* spp. and *Edwardsiella* spp. isolated from the internal organs of cultivated *Rana catesbeiana*, respectively. Similarly, all isolates from reared American bull frogs showed resistance to lincomycin (92%) and high sensitivity to florfenicol and doxycycline (Tee and Najiah, 2011). Our findings of high resistance (93.3-100%) to lincomycin and high sensitivity to doxycycline (90.3%) and florfenicol (75%) were close to these investigations (Table 5).

The MAR index values of all isolates were ranging from 0.13 to 0.73. These values were changing between 0.27-0.73 for Gram negative rod-shaped bacteria, 0.13-0.25 for Gram positive rod-shaped sporeforming bacteria and 0.15-0.47 for Gram positive cocci-shaped non-sporeforming bacteria (Table 6). Although, no antibacterial medication was applied during this study, except enrofloxacin once in the larval stage, the MAR index values of 26 of 31 (83.9%) isolate species were quite high, especially all Gram negative rod-shaped bacteri were higher than 0.20 (100%) (Table 6). Yet, high sentivity (97.8%) of enrofloxacin was detected for Gram negative rod-shaped bacteri (Table 5). MAR index results for *Aeromonas* spp. and *Edwardsiella* spp. (0.45 and 0.33) (Table 6) showed similarity to Lee et al. (2009) (0.27 and 0.31, respectively). Antibiogram and MAR values of Gram negative bacteria detected from wild

frogs have been studied previously in Turkey (Hacioglu and Tosunoglu, 2014; Hacioglu et al., 2015). Hacioglu and Tosunoglu (2014) reported 6.5-46.6% resistance for all Gram negative bacteria and detected 35%, 28%, 19%, and 6% resistance for erythromycin, amoxicillin, oxytetracycline and gentamicin, respectively. Hacioglu et al. (2015) determined the resistance values of the same drugs as 60-85%, 50-80%, 25-55%, and 10-20%. The results of the present study for Gram negative rod-shaped bacteri were 0-97.8% in general, and showed 68.9%, 75.6%, 33.3%, and 20% resistance of the reported drugs, respectively (Table 5). These findings were higher than the values of Hacioglu and Tosunoglu (2014), but congruent to Hacioglu et al. (2015). These inconsistencies can be caused by the variety of frog species, habitat and environment conditions. Whereas MAR index values of Gram negative bacteria of wild frogs were between 0-0.58 (Hacioglu and Tosunoglu, 2014), higher values (0.27-0.73) were detected in reared marsh frogs in our study.

It was pointed out, that reptiles and amphibians, which harbor highly antimicrobial resistant bacteria, can threaten the health of aquatic animals and humans (Hacioglu and Tosunoglu, 2014). Although there was no regular application of drugs in the marsh frog farm, the reasons for the high antibacterial resistance have to be investigated seriously.

CONCLUSION

In this study, presence of opportunistic pathogenic bacteria in frog, water and feed and specimens have been detected in the marsh frog farm in Mersin. Most of these bacteria were previously reported as agents of some diseases of vaious frog species. Some are also zoonotic and can pose risk for even human health. As far as it is known, this is the first microbiological study on farmed marsh frogs. Further investigations are necessary for the development of sustainable marsh frog culture and for protection of health, both frog and human.

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