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Original Research Article

THE PRESENCE OF PUTATIVE VIRULENCE DETERMINANTS, TETRACYCLINE AND β - LACTAMS RESISTANCE GENES OF *Aeromonas* SPECIES ISOLATED FROM PET TURTLES AND THEIR ENVIRONMENT

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Abstract: This study aimed to characterize *Aeromonas* spp. isolated from ten popular species of pet turtles and their environment to evaluate the potential risk of pet turtles as a source of virulence-associated genes, and tetracycline and β-lactams resistance determinants. Presence of eight virulence genes (*ser, aer, exu, lip, fla, ascV, ahyB* and *gcat*), and tetracycline (*tetA, tetB* and *tetE*) and β-lactams (bla_{TEM}, bla_{SHV}, bla_{OXA} and bla_{CTX-M}) resistance genes were evaluated by conventional PCR assays. The *aerA* gene showed the highest frequency of occurrence (92%), followed by *fla* (75%), *gcaT* (68%), *ahyB* (59%), *ser* (39%), *lip* (37%) and *ascV* (25%) genes. None of the isolates carried amplicon of DNase-associated *exu* gene. *A. hydrophila, A. dharkensis, A. veronii* and *A. caviae* were carried seven tested virulence genes except for exu while only four virulence genes were detected in *A. enteropelogenes*. Among the 75 tetracycline-resistant isolates, *tetA, tetE* and *tetB* genes were detected in 38, 26 and 6 isolates, respectively. Among the tested β-lactam resistance genes, bla_{OXA} and bla_{TEM} genes were detected in 54% and 36% of β-lactam resistant isolates, respectively. No bla_{CTX-M} and bla_{SHV} genes were detected. Our results indicate that pet turtle-associated aeromonads, exhibiting potential virulence and antimicrobial (tetracycline and β-lactams) resistance genes, may pose a serious health risk to pet turtle owners, particularly to immunocompromised individuals.

Key words: *Aeromonas* spp.; virulence-associated genes; tetracycline resistance; β-lactams resistance; pet turtle

Introduction

Mesophilic aeromonads are ubiquitous bacteria that are a component of the normal microbiota of many aquatic animals such as fish, amphibians, and reptiles (1). They can cause ulcerative stomatitis, pneumonia, dermatitis, and septicemia in reptiles under stressful conditions such as trapping, handling and temperature variations of rearing environment (2, 3). Over the years, many studies have been investigated to evaluate the prevalence of *Aeromonas* species in aquatic animals, mainly food-producing animals (4, 5). However, a limited number of studies evaluating the distribution of aeromonads in pet turtles have been published up to date (6, 7).

The pathogenesis of *Aeromonas* species involves various virulence factors including cytotoxic heat-labile enterotoxin (*act*), cytotonic heat-labile enterotoxin (*alt*) and cytotonic heat-stable enterotoxin (*ast*), aerolysin (*aer*), lipase (*lip*), serine protease (*ser*), elastase (*ahyB*), DNase (*exu*), glycerophospholipid-cholesterol acyltransferase (*gcaT*), flagellar system (*fla*) and Type III secretion system (TTSS) effector (*ascV*). These genes encoding virulence factors have been broadly used in determining the potential pathogenicity of *Aeromonas* species isolated from the environment, foodstuffs, and human clinical samples (1, 8-10).

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Recently, antibiotic-resistant aeromonads have been recognized as a serious concern due to their potential health risks to animals and humans (11, 12). Especially, the dissemination of tetracycline and β-lactams resistance aeromonads in the aquatic environment has been widely documented (12, 13, 14). Among many tetracycline resistance genes, the *tetE, tetA* and *tetB* genes were frequently identified from *Aeromonas* species in the aquatic environment (12, 15, 16). *Aeromonas* species can produce numerous β-lactamases for conferring resistance to β-lactams. According to isolation sources, the previous studies have shown the different prevalence of genes encoding β-lactamases in *Aeromonas* species. In the aquatic environment, the bla_{TEM}, bla_{SHV}, bla_{OXA} and bla_{CTX-M} β-lactams genes were frequently detected from *Aeromonas* species (17-19).

These resistance genes containing plasmids and transposons are known as mobile genetic elements that can be transferred horizontally among distantly related lineages. Particularly, The aquatic environment is more favorable for the transmission of resistant bacteria, thus, *Aeromonas* species as opportunistic pathogens might be dangerous vectors for the spreading of antibiotic resistance genes through the aquatic environment (18, 20). Hence, the present study was conducted to determine the occurrence of antimicrobial resistance genes (tetracyclines and β-lactams) and virulence-associated genes of *Aeromonas* species isolated from pet turtles and their environment.

Materials and methods

Bacterial isolates

One hundred and two *Aeromonas* species isolates obtained from ten commercially popular pet turtles species (Chinese stripe-necked turtles *Ocadia sinensis*, yellow belly sliders *Trachemys scripta scripta*, river cooters *Pseudemys concinna concinna*, northern Chinese softshell turtles *Pelodiscus maackii*, western painted turtles *Chrysemys picta belli*, peninsula cooters *Pseudemys peninsularis*, African sideneck turtles *Pelusios castaneus*, common musk turtles *Sternotherus odoratus*, red belly cooters *Pseudemys rubriventris* and alligator snapping turtles *Macroclemys Temminckii*) and their rearing environment was screened to investigate the presence of putative virulence, and β-lactams and tetracycline resistance genes. These isolates have been previously characterized for their antimicrobial susceptibilities, enterotoxin (*act*, *alt* and *ast*) genes and quinolone resistance determinants (7, 21).

Detection of antibiotic resistance genes

Twenty-eight and seventy-five isolates were selected (21) for the detection of β-lactams and tetracycline resistance determinants, respectively. These isolates were tested by PCR assays to detect the genetic determinants associated with resistance to β-lactams (bla_{TEM}, bla_{SHV}, bla_{OXA} and bla_{CTX-M}), and tetracyclines (*tetA, tetB* and *tetE*). The primer sets used in PCR amplification are summarized in table 1. PCR amplifications were conducted in 20 µL volumes consisting of 10 µL of Quick Taq® HS DyeMix (Toyobo, Japan), 1 µL of 10 pmol/µL each primer and 1 µL of the template under standard conditions. The PCR products were analyzed by electrophoresis on 2% (wt/vol) agarose gels. Positive controls were implemented with previously characterized enterobacterial strains that harbored the corresponding genes (21, 22).

Detection of virulence-associated genes

All isolates were subjected to PCR assays to detect the 8 tested virulence genes including *ser, aer, exu, lip, fla, ascV, ahyB* and *gcat*. The PCR amplification of the virulence-associated genes was carried out according to the PCR primers and conditions reported previously (Table 1). The PCR mixture of 20 µL contained 10 µL Quick Taq HS DyeMix (Toyobo, Japan), 7 µL PCR water, 1 µL template and 1 µL of each primer. The PCR products were examined by electrophoresis on 1.5% (W/V) agarose gel.

Results

Bacterial isolates

One hundred and two *Aeromonas* species isolates were isolated from the feces, skin and rearing environments of pet turtles and identified by biochemical and *gyrB* sequence analyses. *Aeromonas enteropelogenes* was the predominant species among the isolates (52.9%) followed by *A. hydrophila* (32.4%), *A. dharkensis* (5.9%), *A. veronii* (4.9%) and *A. caviae* (3.9%) [7].

Presence of resistance genes

Among the tested β-lactam resistance genes, bla_{OXA} and bla_{TEM} genes were detected in 54% and 36% of β-lactam resistant isolates, respectively. No bla_{CTX-M} and bla_{SHV} genes were detected (Table 2). Among the 75 tetracycline-resistant isolates, *tetA, tetE and tetB genes* were detected in 38, 26 and 6 isolates, respectively (Table 3).

Table 1: Oligonucleotide primers and PCR conditions [a] used to amplify virulence and antibiotic resistance genes of *Aeromonas* spp.

Gene	Target	Nucleotide Sequence (5'-3')	Size (bp)	Annealing temperature (°C)	Reference
aerA	Aerolysin	F: CTATGGCCTGAGCGAGAAG	431	62	30
		R: CAGTTCCAGTCCCACCACT			
ser	Serine protease	F: ACCGAAGTATTGGGTCAGG	350	55	13
		R: GCTCATGCGTAACTCTGGT			
fla	Flagella	F: CCAACCGTYTGACCTC	608	56	36
		R: MYTGGTTGCGRATGGT			
ahyB	Elastase	F: CACGGTCAAGGAGATCAAC	513	58	13
		R: GCTGGTGTTGGCCAGCAGG			
lip	Lipase	F: ATCTTCTCCGACTGGTTCGG	382	62	36
		R: CCGTGCCAGGACTGGGTCTT			
exu	DNase	F: AGACATGCACAACCTCTTCC	323	59	13
		R: GATTGGTATTGCCTTGCAAG			
gcaT	Glycerophospholipid-cholesterol acyltransferase	F: TCCTGGAATCCCAAGTATCAG	237	65	13
		R: GCAGGTTGAACAGCAGTATCT			
ascV	Type III Secretion System	F: AGCAGATGAGTATCGACGG	891	58	38
		R: AGGCATTCTCCTGTACCAG			
bla_{TEM}	β - lactams resistance	F: ATAAAATTCTTGAAGACGAAA	1080	60	22
		R: GACAGTTACCAATGCTTAATC			
bla_{SHV}		F: TTATCTCCCTGTTAGCCACC	795	60	
		R: GATTTGCTGATTTCGCTCGG			
bla_{CTX-M}		F: CGCTTTGCGATGTGCAG	550	52	
		R: ACCGCGATATCGTTGGT			
bla_{OXA}		F: TCAACTTTCAAGATCGCA	591	60	
		R: GTGTGTTTAGAATGGTGA			
tetA	Tetracycline resistance	F: GTAATTCTGAGCACTGTCGC	1000	62	22
		R: CTGCCTGGACAACATTGCTT			
tetB		F: CTCAGTATTCCAAGCCTTTG	400	57	
		R: CTAAGCACTTGTCTCCTGTT			
tetE		F: GTGATGATGGCACTGGTCAT	1100	62	
		R: CTCTGCTGTACATCGCTCTT			

[a] PCR thermocycle conditions for each reaction; initial denaturation of 94 °C for 2 min followed by a total of 35 cycles of amplification. Each cycle consisted of 94 °C denaturation for 30 s, annealing for 50 s and 72 °C extension for 10 min.

Table 2: β-lactams resistance profiles of turtle-associated *Aeromonas* spp.

Isolate	Host [a]	β-lactam resistance [b]	β-lactam resistance genes
Aeromonas caviae			
AD14	CSN	AMP, AMX, CEP, FOX	bla_{TEM}
AC50	RC	AMP, AMX, CEP, CRO, FOX, IMI	bla_{OXA}, bla_{TEM}
A. dharkensis			
AD17	RC	AMP, AMX, CEP, CRO, FOX, CTX	bla_{OXA}, bla_{TEM}
AD18	RC	AMP, AMX, CEP, CRO, FOX, CTX	bla_{OXA}, bla_{TEM}
AD19	RC	AMP, AMX, CEP, CRO, FOX	bla_{OXA}
AD15	CSN	AMP, AMX, CEP, FOX, CTX	bla_{OXA}
A. enteropelogenes			
AC2	RC	AMP, AMX, CEP	bla_{OXA}
AC6	RC	CEP, FOX	-
AC15	NCS	AMP, AMX, CEP, FOX	bla_{OXA}
AC30	CM	CEP, CTX, ATM	-
AC31	WP	CEP, CTX, ATM	-
AC32	WP	CEP, CTX, ATM	-
AC35	RC	CEP, CTX, ATM	-
AC44	YB	AMP, AMX, CRO	bla_{TEM}
AC45	RC	CEP, CRO, ATM	-
AC53	WP	AMP, AMX, CEP, FOX	bla_{OXA}
AV4	RC	AMP, AMX, CEP, FOX	bla_{OXA}
AD1	CM	AMP, AMX, CEP, FOX	bla_{OXA}
A. hydrophila			
AH1	RC	AMP, CEP	-
AH11	CSN	AMP, AMX, CEP, CRO	bla_{OXA}, bla_{TEM}
AH13	CSN	CEP, CRO, FOX, IMI	-
AH19	NCS	AMP, AMX, CEP, CRO	bla_{OXA}
AH20	NCS	AMP, AMX, CEP	bla_{TEM}
AH22	YB	AMP, AMX, CEP	-
AH23	YB	AMP, AMX, CEP, CRO	bla_{OXA}
AH25	CM	AMP, AMX, CEP, FOX	bla_{TEM}
AD10	AF	AMP, AMX, CEP, FOX	bla_{OXA}, bla_{TEM}
A. veronii			
AC52	SN	AMP, AMX, CEP, FOX	bla_{OXA}, bla_{TEM}

[a]**Host:** CSN= Chinese stripe-necked turtle, YB= yellow belly slider, RC= river cooter, PC= peninsula cooter, NCS= northern Chinese softshell turtle, CM= common musk turtle, WP= western painted turtle, AF= African sideneck turtle, SN= Alligator snapping turtle.
[b]**β-lactams resistance:** AMX=Amoxicillin (10 µg), AMP=Ampicillin (10 µg), CEP=Cephalothin (30 µg), CRO=Ceftriaxone (30 µg), FOX=Cefoxitin (30 µg), CTX=Cefotaxime (30 µg), IMI=Imipenem (10 µg)

Table 3: Distribution of tetracycline resistance genes among tetracycline resistant Aeromonas species isolated from pet turtles and their environment

Species	Number of positive isolates (Subtotal %)		
	tetA	*tetB*	*tetE*
Aeromonas enteropelogenes (*n* = 50)	32 (64)	-	8 (2)
A. hydrophila (*n* = 17)	6 (35)	-	12 (71)
A. dharkensis (*n* = 4)	-	2 (50)	3 (75)
A. veronii (*n* = 3)	-	3 (100)	2 (66)
A. caviae (*n* = 1)	-	1 (100)	1 (100)
Total (%) (*n* = 75)	38 (51)	6 (1)	26 (35)

Table 4: Prevalence of virulence-associated genes in *Aeromonas* species isolates from pet turtles and their environment

Species	Number of positive isolates (Subtotal %)							
	aerA	*lip*	*ahyB*	*ser*	*exu*	*fla*	*gcat*	*ascV*
Aeromonas enteropelogenes (*n* = 54)	46 (85)	0	21 (39)	0	0	51 (94)	23 (43)	0
A. hydrophila (*n* = 33)	33 (100)	30 (91)	28 (85)	31 (94)	0	19 (58)	33 (100)	15 (47)
A. dharkensis (*n* = 6)	5 (83)	3 (50)	4 (67)	4 (67)	0	2 (33)	6 (100)	4 (67)
A. veronii (*n* = 5)	5 (100)	3 (60)	5 (100)	3 (60)	0	2 (40)	4 (80)	3 (60)
A. caviae (*n* = 4)	4 (100)	2 (50)	2 (50)	2 (50)	0	3 (75)	3 (75)	4 (100)
Total (%) (*n* = 102)	93 (92)	38 (37)	60 (59)	40 (39)	-	77 (75)	69 (68)	26 (25)

Distribution of virulence-associated genes

The occurrence and frequencies of virulence genes are shown in Table 4. The *aerA* gene showed the highest frequency of occurrence (92%), followed by *fla* (75%), *gcaT* (68%), *ahyB* (59%), *ser* (39%), *lip* (37%) and *ascV* (25%) genes. None of the isolates carried amplicon of the DNase-associated *exu* gene.

Discussion

The *Aeromonas* spp. under study were multi-drug-resistant turtle-associated bacteria which carried quinolone resistance determinants, as well as enterotoxin genes (7, 21). The isolates were highly resistant to β-lactams especially amoxicillin, ampicillin and cephalothin. β-lactam antibiotics have used for the treatment of *Aeromonas* infection during the last decade. However, their efficacy has significantly declined due to the production of β-lactamases by resistant bacterial strains (14, 17, 23). The *Aeromonas* spp. are naturally resistant to β-lactams because of the expression of chromosomal β-lactamases (24).

In this study, twenty-eight aeromonads isolates were resistant to the more than one β-lactam antibiotics. Among them, 54% and 36% of isolates harbored bla_{OXA} and bla_{TEM} genes. Several previous studies have documented the detection of the bla_{OXA} and bla_{TEM} genes in *Aeromonas* isolates recovered from the environment (14, 25) and clinical samples (26) and the prevalence of gene detection varies according to the isolation sources. In Korea, a previous study reported that the bla_{OXA} and bla_{TEM} genes were detected in 3% and 100% of *Aeromonas* isolates from aquaculture fish [14]. However, a different trend was observed in this study which the bla_{OXA} and bla_{TEM} genes were detected in *Aeromonas* isolates from pet turtles that suggest a wide distribution of β-lactamase genes in *Aeromonas* isolates from various sources.

A much higher level of tetracycline resistance was observed amongst aeromonads in our previous study (7) and 78 of tetracycline-resistant isolates were selected to detect their tetracycline resistance determinants (*tetA, tetB* and *tetE*). *A. enteropelogenes* and *A. hydrophila* harbored *tetA* and *tetE* genes while other *Aeromonas* species harbored *tetB* and *tetE* genes. Previous reports indicate that the *tetA* and *tetE* determinants are the predominant tetracycline resistance genes in the aquatic environment (16, 27) and both genes code for an efflux pump that eliminates the drug from the cell [28]. The *tetA, tetB* and *tetE* genes are located on the plasmid as well as *tetA* in the transposon (Tn*1721)* and *tetE* is adjacent to the integrons (15). Han et al. (27) has reported that *tetE* gene was the predominant tetracycline determinant in *Aeromonas* spp. isolated from Korean fish farms and aquariums. However, Kim et al. (29) reported that *tetA* was the most frequent gene in *A. salmonicida* strains isolated from salmonid farms and private aquariums in Korea. The *tetB* gene was detected at a low frequency, while Jacobs and Chenia. (12) reported a lower prevalence of *tetB* genes among *Aeromonas* spp. isolated from the South African aquaculture system.

Detection of virulence encoding genes of *Aeromonas* spp. have been widely applied for evaluating their potential pathogenicity (30, 31). However, the prevalence of virulence-associated genes has rarely been reported in *Aeromonas* strains from pet turtles (7). In the current study, *Aeromonas* isolates were found to possess genes *aerA, lip, ahyB, ser, fla, gcat* and *ascV*, while genes for DNase (*exu*) was not identified. Especially, none of *A. enteropelogenes* isolates harbored *lip, ser, exu* and *ascV*

genes. Previous studies have revealed that multiple virulence-associated genes are present in *Aeromonas* isolates and having high heterogeneity in the distribution of virulence-associated genes (10, 30, 31). The pore-forming aerolysin/hemolysin encoded *aer* gene was the most prevalent in this study which was detected in 92% of the total isolates representing all species of the genus. Several studies have reported the high prevalence of the *aer* gene in clinical and environmental *Aeromonas* isolates (30, 32).

The three enterotoxins *act, alt*, and *ast* have been implicated as major virulence factors in diarrhoeal disease which had been investigated in our previous study (7). However, the presence of these toxins might not be enough for virulence (31). The temperature-stable metalloprotease with elastolytic activity (*ahyB*) and serine protease (*ser*) play an important role in the invasiveness and establishment of infection (1). In the current study, the *ahyB* and *ser* genes were detected in 59% and 39% of isolates, respectively. None of the *A. enteropelogenes* isolates harbored *ser* gene. The flagella are important appendages for the initial attachment of bacteria to the gastrointestinal epithelium and involve in the subsequent adherence process and biofilm formation (33, 34). The *fla* gene-encoded polar flagella were common among the *Aeromonas* isolates from the aquatic environment. The *fla* gene was detected in 99% of *Aeromonas* isolates from diseased eel in Korea (10). The *gcaT* gene plays a coherent, integrated role in the establishment of pathogenicity of *Aeromonas* spp. by involving in the regulation and secretion of extracellular glycerophospholipid-cholesterol acyltransferase (13). The *gcaT* gene was detected in 68% of *Aeromonas* isolates.

Lipases play a role as virulence factors by interacting with leukocytes or by disturbing several immune system functions through free fatty acids produced by the lipolytic activity. Extracellular lipases secreted by *Aeromonas* spp. actively involve in the alteration of the host plasma membrane and thus increase the severity of infection (35). Among *Aeromonas* strains isolated in the present study, 91% of *A. hydrophila*, 60% of *A. veronii*, 50% of *A. dharkensis* and 50% of *A. caviae* isolates were found to have *lip* gene. Several previous studies reported a high prevalence of *lip* gene among the *Aeromonas* isolates from the aquatic environment (10, 36). Type III secretion system (T3SS) plays a crucial role in host-pathogen interactions by injecting effector toxins directly into the cytosol of host cells (37). The *acsV* gene encodes the T3SS and which was detected in 59% of *Aeromonas* spp. except for *A. enteropelogenes* isolates. The presence of *ascV* gene was previously detected in 68% of *Aeromonas* spp. isolated from diseased farmed fish and farm environment (38). Besides, the high frequency of *ascV* gene was reported in human clinical isolates (37).

The *exu* gene is responsible for DNA hydrolysis which was not detected in this study. The absence of *exu* gene was also reported by Nawaz et al. (13) in *A. veronii* isolated from catfish in the USA. In contrast, the high prevalence of *exu* gene was observed in *Aeromonas* spp. isolated from freshwater lakes in Malaysia (39) and diseased eel in South Korea (10). The specificity of the host or environmental source could be the possible reasons for the absence of *exu* gene in this study.

According to the available literature, this is the first description of these virulence-associated genes in *Aeromonas* of pet turtle origin. Most of *Aeromonas* strains isolated from pet turtles and their environment harboring multiple virulence-associated genes have the potential to be pathogenic. Turtle born aeromonads carrying tetracycline and β-lactams resistance determinants can disseminate through the environment. Collectively, which may pose a public health risk to pet turtle owners, particularly to immunocompromised individuals.

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PRISOTNOST DETERMINANT ZA DOLOČITEV DOMNEVNE VIRULENCE TER GENOV ZA ODPORNOST NA TETRACIKLIN IN β-LAKTAM VRST *Aeromonas* IZOLIRANIH IZ LJUBITELJSKIH VRST ŽELV IN IZ NJIHOVEGA OKOLJA

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Izvleček: Namen študije je bil določiti bakterije *Aeromonas* spp., izolirane iz desetih priljubljenih vrst hišnih želv in njihovega okolja, z namenom ocenjevanja potencialnega tveganje hišnih želv kot vira genov, povezanih z virulenco, ter determinante odpornosti proti tetraciklinom in β-laktamom. Prisotnost osmih virulentnih genov (*ser, aer, exu, lip, fla, ascV, ahyB in gcat*) ter genov za odpornost na tetracikline (*tetA, tetB in tetE*) in β-laktame (bla_{TEM}, bla_{SHV}, bla_{OXA} in bla_{CTX-M}) je bila ocenjena s konvencionalnimi testi PCR. Najbolj pogost je bil Gen *aerA* (92 %), sledili so geni *fla* (75 %), *gcaT* (68 %), ahyB (59 %), ser (39 %), lip (37 %) in *ascV* (25 %). Nobeden od izolatov ni imel pomnoženega gena *exu*, povezanega z DNAzo. *A. hydrophila*, *A. dharkensis*, *A. veronii* in *A. caviae* so vsebovali sedem testiranih genov virulence, razen *exu*, medtem ko so bili v *A. enteropelogenih* odkriti le štirje virulenčni geni. Med 75 izolati, odpornimi na tetracikline, so bili geni *tetA*, *tetE* in *tetB* odkriti v 38, 26 oziroma 6 izolatih. Med preizkušenimi geni za odpornost proti β-laktamu so bili geni bla_{OXA} in bla_{TEM} odkriti pri 54 % oziroma 36 % izolatov, odpornih proti β-laktamu.

V nobenem vzorcu nista bila zaznana gena bla_{CTX-M} in bla_{SHV}. Rezultati študije kažejo, da bakterije *Aeromonas spp.* iz hišnih želv lahko imajo potencialne virulenčne gene in gene za odpornost proti tetraciklinu in β-laktamom, in lahko potencialno ogrožajo zdravje lastnikov hišnih želv, zlasti imunsko oslabljenih posameznikov.

Ključne besede: *Aeromonas* spp.; geni povezani z virulenco; odpornost na tetracikline; rezistenca na β-laktami; ljubiteljske vrste želv