Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Antimicrobial Resistance and Virulence Factors of *Edwardsiella tarda* Isolated from Pet Turtles

Dong-Min Shin[§], Sabrina Hossain[§], SHMP Wimalasena and Gang-Joon Heo*

Veterinary Medical Center and College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Korea
*Corresponding author: gjheo@cbu.ac.kr

ARTICLE HISTORY (16-190)

Received: July 24, 2016
Revised: October 08, 2016
Accepted: October 14, 2016
Published online: November 17, 2016

Key words:
Antimicrobial resistance
Edwardsiella tarda
Pet turtles
Virulence genes *citC*
wecC

A B S T R A C T

Pet turtles are known as a source of bacterial infection to humans when handled in captivity. Pathogenic *Edwardsiella tarda* was identified using 16S rRNA sequencing, and characterized using conventional PCR analysis with *E. tarda*-specific virulence primer sets and antimicrobial susceptibility tests with a disk diffusion test. *E. tarda* was isolated from 12 fecal samples of 27 commercially popular pet turtles purchased through pet shops and online markets in Korea. All isolates were confirmed as *E. tarda* through biochemical analysis and 16S rRNA sequencing. PCR analysis showed that the virulence genes *citC* and *wecC* were present in all isolates and indicated their potential pathogenicity. The strains showed susceptibility against amikacin, amoxicillin, cefoxitin, ceftriaxone, ciprofloxacin, gentamicin, imipenem and streptomycin but were resistant to colistin and trimethoprim/sulfamethoxazole in disk diffusion test. Intermediate resistance was noted against ampicillin and nalidixic acid. Most isolates displayed resistance against tetracycline. These results indicate that pet turtles pose a potential risk of exposure to a zoonotic pathogen in humans from a public health standpoint.

To Cite This Article: Shin DM, Hossain S, Wimalasena SHMP and Heo GJ, 2017. Antimicrobial resistance and virulence factors of *Edwardsiella tarda* isolated from pet turtles. Pak Vet J, 37(1): 85-89.

INTRODUCTION

Edwardsiella tarda, a gram negative intracellular bacterium, is frequently isolated from reptiles such as crocodiles (*Crocodylus acutus* and *C. moreletti*) (Charruau *et al*., 2012), American alligators (*Alligator mississippiensis*) (Johnston *et al*., 2010) and Geoffroy's toadhead turtles (*Phrynops geoffroanus*) (Ferronato *et al*., 2009). *E. tarda* is also part of the normal flora of the digestive tract of reptiles. Aside from the healthy carrier status of diverse reptile species, septicemia associated with *E. tarda* was described in captive crocodiles (Buenviaje *et al*., 1994). Prior studies have reported multiple subcutaneous abscesses in a caged grass snake (*Natrix natrix*) (Kobolkuti *et al*., 2013).

E. tarda is known as unusual human pathogen, but it has also been associated with several manifestations in humans. *E. tarda* has been collected from samples of human feces, blood, urine, cerebrospinal fluid, bile, peritoneal fluid, and lesions. It has also instigated several diseases in humans including gastroenteritis, meningitis, cholecystitis, endocarditis, osteomyelitis, myonecrosis, soft tissue infections, bacteremia and septicemia (Slaven *et al*., 2001; Nelson *et al*., 2009). In most cases, the presence of certain virulence factors such as virulence genes facilitates them to cause infections in humans (Srinivasa *et al*., 2003).

Contact with or eating undercooked aquatic animals such as fish infected with *E. tarda* is considered an infection route of *E. tarda* to humans (Leung *et al*., 2012). Those most affected tend to be compromised individuals with underlying diseases such as hepatobiliary diseases, malignancy, or diabetes (Nelson *et al*., 2009). The reservoir and source of infections are usually linked to aquatic habitats and aquatic animals (Tsuji *et al*., 2008; Nelson *et al*., 2009; Schlenker and Surawicz, 2009).

Recently, along with the flourishing companion animal industry the pet reptile business has gained worldwide popularity. However, reptiles are known to be asymptomatic carriers of various enteric bacteria associated with warm-blooded animals (Jang *et al*., 2008). The bacteria can be transmitted by physical contact with the infected turtles or through contaminated environments such as water and soil in turtle cages. Since 1975, the US government illegalized selling or distributing small turtles with a carapace less than 4 inches long specially to prevent young children from putting them in their mouth (Harris *et al*., 2010). This ban is a strong public heath measure to prevent zoonotic transmission of numerous bacteria associated with pet turtles (Ebani and Fratini,

2005); however, these regulations do not exist in other first-world countries such as Korea.

This research aims to identify and characterize pathogenic *E. tarda* in pet turtles purchased from pet shops and online markets to determine the potential risk of exposure to a zoonotic pathogen from a public health standpoint and provide information concerning the prevention of transmission to humans.

MATERIALS AND METHODS

Purchase of pet turtles: Twenty-seven turtles of eight commercially popular species were purchased through pet shops and online markets in Korea. Among the 27 turtles, 3 Chinese stripe-necked turtles (*Ocadia sinensis*), 5 yellow belly sliders (*Trachemys scripta scripta*), 6 river cooters (*Pseudemys concinna concinna*), 2 northern Chinese softshell turtles (*Pelodiscus maackii*), 3 western painted turtles (*Chrysemys picta belli*), 3 peninsula cooters (*Pseudemys peninsularis*), 3 common musk turtles (*Sternotherus odoratus*) and 2 African sideneck turtles (*Pelusios sinuatus*) were studied.

Fecal sample collection: Each of the purchased turtles was placed in separate 500 mL beakers with 5 mL of sterilized distilled water for 24 h. One mL of the distilled water containing a turtle's feces was taken as a fecal sample.

Enrichment and isolation of *E. tarda*: The fecal samples were enriched in tetrathionate broth (TTB) (MBcell Ltd., Korea) at 27°C for 24 h. The enriched samples were spread onto *Edwardsiella tarda* (ET) agar (Castro *et al*., 2011) or *Salmonella Shigella* (SS) agar (MBcell Ltd., Korea), for comparison. These plates were incubated at 27°C for 48 h.

Phenotypic identification: Presumptive typical colonies such as white colonies with a black center on ET and SS agar were inoculated on triple sugar iron agar (TSI) (MBcell Ltd., Korea) and motility-indole-lysine medium (MIL) (MBcell Ltd., Korea) and incubated at 27°C for 48 h. Any isolates exhibiting an alkaline slant and acid butt with H2S and gas in TSI agar and indole and lysine decarboxylase production in MIL medium were selected for additional testing. Suspect isolates were tested for citrate utilization in Simmons citrate agar; methyl red and Voges-Proskauer (VP) reactions in methyl red and VP broth; acid production from mannitol, maltose and glucose in 0.5% in phenol red broth; and ornithine dihydrolase activity in motility-indole-ornithine medium.

16S rRNA sequencing and detection of virulence genes by PCR: All 12 presumptive *E. tarda* strains were sent to Cosmogenetech, Co., Ltd. (Korea) for 16S rRNA sequencing with the universal primers 27F and 1492R. A neighbor-joining phylogenetic tree was prepared based on 16S rRNA gene sequences. In addition, the polymerase chain reaction (PCR) was used to detect the presence of *citC* and *wecC* genes in the sequenced strains. The primers used are shown in Table 1, and PCR was conducted by Cosmogenetech, Co., Ltd. The PCR mixture contained PCR buffer (10 mM Tris/HCl, 50 mM KCL, 1-5 mM $MgCl_2$, pH 8.3), 200 μM of each dNTP, 12.5 pmol of each primer, DMSO at a final concentration of 4%, 1 U Ampli*Taq* DNA polymerase, and 25 ng DNA template. The DNA was amplified in a thermal cycler using the following protocol: 94°C for 3 min, 30 cycles of 94°C for 30 s, 50°C for 1 min and 72°C for 1 min 30 s, and 72°C for 5 min. Each gene was amplified separately. PCR products were separated in a 1% agarose gel for 1 h at 100V, stained with ethidium bromide and detected by UV transillumination.

Antimicrobial resistance testing: All 12 isolates were subjected to disk diffusion testing with 15 common antibiotics for veterinary use against gram-negative bacteria. The testing was carried out according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards (Charles *et al*., 2010). Disks containing amoxicillin (30 μg), ampicillin (10 μg), chloramphenicol (30μg), colistin sulfate (10 μg), gentamycin (10 μg), nalidixic acid (30 μg), tetracycline (30 μg), amikacin (30 μg), cefoxitin (30 μg), ceftriaxone (30μg), cephalothin (30 μg), ciprofloxacin (5 μg), imipenem (10 μg), streptomycin (10 μg) and trimethoprim (1.25 μg)/sulfamethoxazole (23.75 μg) were purchased from Kisan Biotech Co., Ltd. (Korea).

Table 1: Primers used for PCR amplification of virulence factors

Gene	Sequence (5'-3')	Size (bp)
citC-F	TTTCCGTTTGTGAATCAGGTC	596
citC-R	AATGTTTCGGCATAGCGTTG	
wecC-F	CCTTATAAATTACTCGCT	352
wecC-R	TTTGTGGAGTAACAGTTT	

RESULTS

Isolation of *E. tarda* from fecal samples: Suspicious colonies were isolated from 12 of 27 turtle fecal samples (44.4%) (Table 2). Positive samples were from 2 of 3 Chinese stripe-necked turtles, 2 of 5 yellow belly sliders, 5 of 6 river cooters, 1 of 2 Chinese softshell turtles, 1 of 3 common musk turtles and 1 of 2 African sideneck turtles. In both SS and ET agar, all *E. tarda* isolates showed a black center indicating H_2S production. On SS agar, they were translucent and assumed the color of the medium while colonies of *E. tarda* in ET agar appeared clear to whitish (Castro *et al.*, 2011). Furthermore, 12 isolates were positive in the motility, indole and gas production tests but negative in the urea and citrate test.

16S rRNA sequencing and detection of virulence genes by PCR: 16S rRNA sequencing of all isolates confirmed them as *E. tarda*. In phylogenetic tree, all of the isolates from pet turtles were clustered with *E. tarda* in a group (Fig. 1). In addition, as shown in Fig. 2, PCR results clearly indicated the presence of the virulence genes *citC* (596 bp) and *wecC* (352 bp) required for human penetration activity.

Antimicrobial resistance testing: The results of the disk diffusion test are shown in Table 3. Clinical and Laboratory Standards Institute standards (Charles *et al*., 2010) were used to determine the efficacy of each antibiotic. Most isolates were susceptible to the majority of commonly used veterinary antibiotics against gram-negative bacteria including amikacin, amoxicillin, cefoxitin, ceftriaxone,

Table 2: Isolation rates of *E. tarda* from the fecal samples in turtles

Species	Tested samples	Positive numbers	Isolation rate*(%)
Chinese stripe-necked turtle (*Ocadia sinensis*),	3	2	66.7
Yellow belly slider (*Trachemys scripta scripta*)	5	2	40
River cooter (*Pseudemys concinna concinna*)	6	5	83.3
Northern Chinese softshell turtle (*Pelodiscus maackii*)	2	1	50
Western painted turtle (*Chrysemys picta belli*)	3	0	0
Common musk turtle (*Sternotherus odoratus*)	3	1	33.3
African sideneck turtle (*Pelusios sinuatus*)	2	1	50
Peninsula cooter (*Pseudemys peninsularis*)	3	0	0
Total	27	12	44.4

* Isolation rate: the ratio of positive samples to total samples.

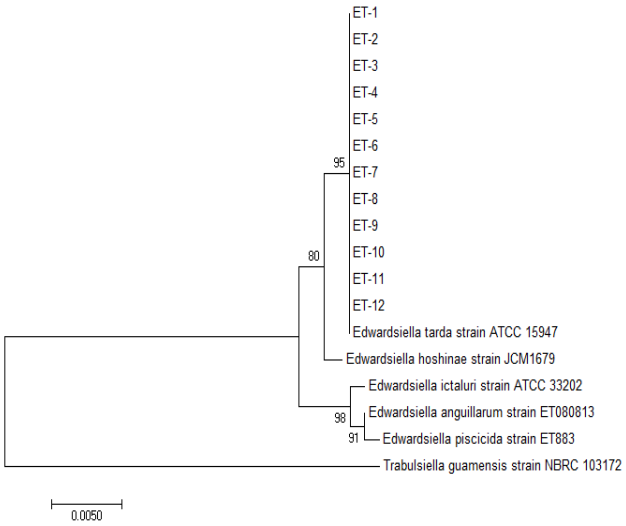

Fig. 1: Phylogenetic tree based on 16S rRNA sequence of *E. tarda* isolates from pet turtles. *Trabulseilla guamensis* was used as an outgroup. Bootstrap values were asserted by the percentage of 1,000 replicates shown at the branching points.

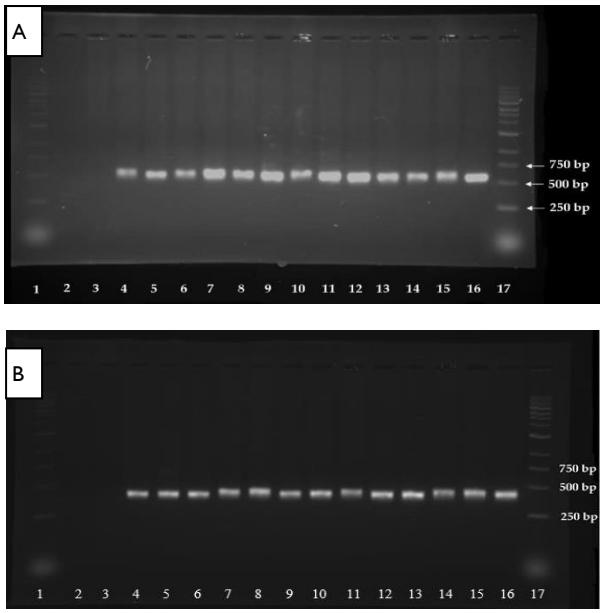

Fig. 2: PCR results of *citcC* (a) and *wecC* (b) virulence genes in *E. tarda* isolates (lanes 5-16), Another lanes for blank control; distilled water (lane 2), negative control; *Aeromonas hydrophila* (lane 3), positive control; *E. tarda* ATCC reference strain (lane 4), and 1kb DNA ladders (lane 1 and lane 17).

ciprofloxacin, gentamicin, imipenem and streptomycin. The strains showed 100% resistance against colistin and trimethoprim/sulfamethoxazole. Intermediate resistance was noted against ampicillin and nalidixic acid. Most isolates displayed resistance against tetracycline, but three isolates presented intermediate resistance.

DISCUSSION

As reptile pet ownership is getting more popular, the need for awareness about associated public health concerns grows. Among pet reptiles, turtles are known as a potential reservoir for zoonotic pathogens, and they often excrete pathogenic bacteria as normal flora (Mitchell, 2011). Recently, the rise in single-person households and elderly households and low birthrate caused an increase in the importation of exotic animals such as insects, turtles, and tropical fish. As the affection between the owner and pet deepens, physical contact allows for emotional sympathy and stability. However, this is accompanied by the risk of exposure to a pathogen (Woodward *et al*., 1997). Additionally, *E. tarda* is commonly isolated from both freshwater and marine environments. Most Korean people have a custom of eating raw fish or shrimp and some species of Chinese Softshell turtles are bred in captivity for consumption (Lo *et al*., 2009). This is the first study to report the isolation of a pathogenic strain of *E. tarda* from commercially popular species of pet turtles.

In this study, we isolated presumptive *E. tarda* from fecal samples of 12 of 27 turtles purchased (44.4%). Contaminated turtles could contaminate the environment as well through feces and shedding. Environmental contamination from fecal samples of one turtle could therefore lead to horizontal transmission, which suggests that pet shops neglecting proper sanitation could increase *E. tarda* contamination (Hirai *et al*., 2015). This result indicates that raising and distributing pet turtles carries an associated risk of *E. tarda* contamination.

16S rRNA sequencing confirmed all isolates as *E. tarda*. In phylogenetic tree, all *E. tarda* isolates were clustered with *E. tarda* reference strain in one group with 95% of bootstrap value. A monophyletic cluster was also found with the isolates and *E. hoshinae* which is closely related to *E. tarda*. A genetic differential analysis through PCR assay found that these isolates also contained the virulence genes *citC* and *wecC*. Prior studies about *E. tarda* genetic differential analysis indicate that the catalase gene *citC* may be a useful marker for detecting virulent *E. tarda* strains. The virulence gene *citC* is one of the five genes in the citrate lyase operon. This operon helps in cleaving citrate to oxaloacetate and acetate (Srinivasa *et al*., 2003). This gene, while not inherently indicative of pathogenicity, encodes pathogenic factors that aid in virulence. Additionally, based on the public

Table 3: Antimicrobial resistance pattern of the isolates by disk diffusion test

Antimicrobials	Antimicrobial inhibition zone diameter (mm)												Antimicrobial susceptibility* (%)		
	ET-1	ET-2	ET-3	ET-4	ET-5	ET-6	ET-7	ET-8	ET-9	ET-10	ET-11	ET-12	R	I	S
Amikacin	18	19	18	17	20	20	20	22	20	20	20	21	0	0	100
Amoxicillin	19	19	19	19	20	22	21	21	18	19	19	19	0	0	100
Ampicillin	14	14	14	14	15	15	14	16	15	14	14	14	0	100	0
Cefoxitin	19	19	20	18	18	19	19	19	18	15	15	20	0	17	83
Ceftriaxone	24	24	24	23	26	20	25	25	25	24	27	25	0	8	92
Cephalotin	18	19	18	19	19	19	15	19	20	21	21	21	0	8	92
Chloramphenicol	19	21	22	22	22	22	19	19	20	20	20	21	0	0	100
Ciprofloxacin	35	35	35	35	38	38	37	38	38	35	35	35	0	0	100
Colistin	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0
Gentamicin	17	17	17	17	20	21	22	20	19	20	21	21	0	0	100
Imipenem	26	25	24	25	25	25	25	21	25	25	27	28	0	8	92
Nalidixic acid	15	15	15	15	14	15	15	15	15	14	15	15	0	100	0
Streptomycin	20	20	19	21	22	25	25	20	21	21	24	20	0	0	100
Tetracycline	8	2	2	13	5	14	14	10	10	7	7	5	75	25	0
Trimethoprim/ sulfamethoxazole	9	5	5	5	5	5	5	5	3	5	4	4	100	0	0

*Inhibition zone diameters were evaluated according to CLSI (2014) standards; R: Resistance; I: Intermediate resistance; S: Susceptibility.

health importance of zoonosis, we were interested in whether the isolated strains were pathogenic to humans. Therefore, the presence of *wecC*, which encodes a protein that mediates motility associated with the penetration of *E. tarda* through human epithelial layers, is vital in determining the possible turtle-to-human transmission of the pathogen (Suezawa *et al*., 2016) . The PCR results in this study, which were positive for both genes, indicate that the isolates from the turtles are virulent strains that are able to penetrate human epithelial cells and potentially cause human infection.

E. tarda is naturally resistant to macrolides, lincosamides, streptogramins, glycopeptides and rifampin (Stock and Wiedemann, 2001), which was in agreement with the results of this study. Furthermore, all strains were resistant to colistin, which is considered to be the most appropriate drug in isolating and detecting presumptive *E. tarda* infection through simple biochemical tests (Castro *et al*., 2011). However, unlike prior studies indicating the pathogen's susceptibility to most antibiotics, *in vitro* activity of three cephalosporins (cephalothin, cefoxitin and ceftriaxone), commonly used drugs against *E. tarda* infections, did not show 100% susceptibility. Furthermore, in contrast to prior indication of *E. tarda*'s natural resistance to tetracycline, strains in this study were mostly resistant. In a recent study, *E. tarda* isolated from olive flounder (*Paralichthys olivaceus*) showed a high degree of resistance to kanamycin, tetracycline, ampicillin, and streptomycin (Jun *et al*., 2004). This result was different from the result in this study, as these isolates showed 100% susceptibility to streptomycin. The strains also appear to be resistant to numerous other veterinary antibiotics that are generally used in treating *E. tarda* infection (Lim *et al*., 2016). These results indicate that examination of natural antibiotic susceptibility patterns in *Edwardsiella* spp. is necessary because very little is known about their antimicrobial susceptibilities (Stock and Wiedemann, 2001). Furthermore, frequent use of antimicrobial agents may result in the generation of bacteria resistant to multiple antibiotics. Thus, public awareness of the risk of contracting or transferring *E. tarda* from pet turtles is integral to prevent further evolution of antimicrobial resistance.

Conclusions: This study concluded that commercially distributed pet turtles in Korea may serve as a potent reservoir for *E. tarda* and that strains that reside in pet turtles may infect humans through contact with pet turtles, which, in combination with the lack of knowledge about antibiotic resistance patterns of *E. tarda*, poses a significant public health risk.

Author's contribution: DMS and GJH conceived and designed the study. DMS and SH executed the experiment and finalized the data. SHMPW helped in sampling. DMS and GJH wrote the manuscript. §DMS and SH contributed equally to this work. All authors read and approved the final manuscript.

REFERENCES

Buenviaje GN, Ladds PW, Melville L and Manolis SC, 1994. Disease-husbandry associations in farmed crocodiles in Queensland and the Northern Territory. Aust Vet J 71:165-73.

Castro N, Toranzo AE, Nuñez S and Magariños B, 2011. Evaluation of the selective and differential ET medium for detection of *Edwardsiella tarda* in aquaculture systems: Evaluation of ET medium for *E. tarda* detection. Lett Appl Microbiol 53:114-9.

Charles PM, Kali A and Srirangaraj S, 2016. Comparison of carbapenem breakpoints in Clinical Laboratory Standard Institute and European Committee on Antimicrobial Susceptibility Testing guidelines on antibiotic susceptibility test reporting of *Acinetobacter baumannii*. Indian J Pathol Microbiol 59:429-30.

Charruau P, Pérez-Flores J, Pérez-Juárez J, *et al*., 2012. Oral and cloacal microflora of wild crocodiles *Crocodylus acutus* and *C. moreletii* in the Mexican Caribbean. Dis Aquat Organ 98:27-39.

Ebani VV and Fratini F, 2005. Bacterial zoonoses among domestic reptiles. Ann Della Fac Med Vet 58:85-91.

Ferronato BO, Marques TS, Souza FL, *et al*., 2009. Microbiota bacteriana oral e lesões traumáticas no cágado *Phrynops geoffroanus* (Testudines, Chelidae) no sudeste do Brasil. Phyllomedusa J Herpetol 8:19.

Harris JR, Neil KP, Behravesh CB, *et al*., 2010. Recent multistate outbreaks of human salmonella infections acquired from turtles:a continuing public health challenge. Clin Infect Dis 50:554-9.

Hirai Y, Asahata-Tago S, Ainoda Y, *et al*., 2015. *Edwardsiella tarda* bacteremia. A rare but fatal water- and foodborne infection: Review of the literature and clinical cases from a single centre. Can J Infect Dis Med Microbiol 26:313-8.

Jang YH, Lee SJ, Lim JG, *et al*., 2008. The rate of *Salmonella* spp. infection in zoo animals at Seoul Grand Park, Korea. J Vet Sci 9:177-81.

Johnston MA, Porter DE, Scott GI, *et al*., 2010. Isolation of faecal coliform bacteria from the American alligator (*Alligator mississippiensis*). J Appl Microbiol 108:965-73.

Jun LJ, Jeong JB, Huh MD and Jeong HD, 2004. Detection of tetracycline-resistance determinants by multiplex polymerase chain reaction in *Edwardsiella tarda* isolated from fish farms in Korea. Aquaculture 240:89-100.

Kobolkuti LB, Czirjak GA, Tenk M, *et al*., 2013. *Edwardsiella tarda* associated subcutaneous abscesses in a captive grass snake (*Natrix natrix*, Squamata:Colubridae). Kafkas Univ Vet Fak Derg 19:1061-3.

Leung KY, Siame BA, Tenkink BJ, *et al*., 2012. *Edwardsiella tarda*-Virulence mechanisms of an emerging gastroenteritis pathogen. Microbes Infect 14:26-34.

Lim YJ, Kim DH, Roh HJ, *et al*., 2016. Epidemiological cut-off values for disc diffusion data generated by standard test protocols from *Edwardsiella tarda* and *Vibrio harveyi*. Aquac Int 24:1153-61.

Lo YC, Hung CC, Lai CS, *et al*., 2009. Human trichinosis after consumption of soft-shelled turtles. Taiwan Emerg Infect Dis 15:2056-8.

Mitchell MA, 2011. Zoonotic diseases associated with reptiles and amphibians:an update. Vet Clin North Am Exot Anim Pract 14:439-56.

Nelson JJ, Nelson CA and Carter JE, 2009. Extraintestinal manifestations of *Edwardsiella tarda* infection:a 10-year retrospective review. J La State Med Soc 161:103-6.

Schlenker C and Surawicz CM, 2009. Emerging infections of the gastrointestinal tract. Best Pract Res Clin Gastroenterol 23:89-99.

Slaven, EM, Lopez FA and Hart SM, 2001. Myonecrosis caused by *Edwardsiella tarda*:a case report and case series of extraintestinal *E. tarda* infections. Clin Infect Dis 32:1430-33.

Srinivasa RPS, Lim TM and Leung KY, 2003. Functional genomics approach to the identification of virulence genes involved in *Edwardsiella tarda* pathogenesis. Infect Immun 71:1343-51.

Stock I and Wiedemann B, 2001. Natural antibiotic susceptibilities of *Edwardsiella tarda*, *E. ictaluri*, and *E. hoshinae*. Antimicrob Agents Chemother 45:2245-55.

Suezawa CM, Yasuda K, Negayama T, *et al*., 2016. Identification of genes associated with the penetration activity of the human type of *Edwardsiella tarda* EdwGII through human colon epithelial cell monolayers. Microb Pathog 95:148-56.

Tsuji A, Hirasawa K, Arakuma T, *et al*., 2008. A 12-year-old boy with acute gastroenteritis caused by *Edwardsiella tarda* O4:H4. J Jpn Soc Chemother 14:433-5.

Woodward DL, Khakhria R and Johnson WM, 1997. Human salmonellosis associated with exotic pets. J Clin Microbiol 35:2786-90.