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Research Article

Frequency and antimicrobial resistance of *Yersinia enterocolitica* isolated from fish meat caught in Anzali Wetland

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Abstract

Yersinia enterocolitica is the cause of yersiniosis in humans and its symptoms range from mild diarrhea to mesenteric lymphadenitis that may lead to appendicitis. In this study, the frequency and antibiotic resistance of *Yersinia enterocolitica* isolated from fish caught in Anzali wetland were investigated. Sampling was carried out for one year in four seasons from the autumn 2018 to the summer 2019 from four geographical directions: east, west, north and south, from different wetland levels. A total number of 91 fish were sampled from the body of these fish. Microbiological tests including biochemical tests, specific culture, and PCR were performed. In the specific culture method, 20 samples (21.97%) were contaminated with *Yersinia enterocolitica.* For the final confirmation of PCR, 20 samples were isolated from 14 samples (15.4%) containing specific culture method (6 Carp, 3 Anchovy, 0 Bream, 4 Pike Perch and 1 Pike) were infected with *Yersinia enterocolitica*. The chi-square test showed that there was no statistically significant difference between the various seasons (p >0.05), while there was a significant difference between fish species (p <0.05). Isolates from *Yersinia enterocolitica* using an antibiogram test of 10 antibiotics, the most susceptible isolates, Imipenem (92.8%), Kanamycin and Gentamicin (85.7%), Ceftazidime (78.57%). Cotrimoxazole and Nalidixic acid (71.4%) were the most resistant isolates to Ampicillin (64.28%), respectively. The results showed high levels of contamination of *Yersinia enterocolitica*, and indicative of potential dangers of *Yersinia enterocolitica* pathogenicity, especially in immunocompromised and susceptible individuals. Further studies on the pathogenicity of *Yersinia enterocolitica* in fish within the Anzali wetland and preventing sewage from entering the wetland are suggested.

Introduction

Yersinia enterocolitica is a Gram-negative and coccobacillus psychrotrophic enterobacteria of the family Enterobacteriaceae (EFSA and ECDC, 2016), isolated from a variety of environmental sources, foods, water contaminated with sewage and human clinical samples (Alexandrino *et al*., 2003; Thoerner *et al*., 2003; Soltan Dallal *et al*., 2004). The symptoms range from mild and self-limiting gastroenteritis to acute enteritis with diarrhea and stomach ache, mesenteric lymphadenitis, and pseudo-appendicular syndromes indistinguishable from acute appendicitis particularly in children older than 5 years, or septicemia in elderly and immunocompromised individuals (Petsios *et al*., 2016; Saraka *et al*., 2017). *Yersinia enterocolitica* causes a human infection known as Yersiniosis the third most commonly reported zoonosis in Europe in 2015, after campylobacteriosis and salmonellosis (EFSA and ECDC, 2015; Rohde *et al*., 2017). The great majority of gastrointestinal infections are self-limiting and confined to the gut and do not require antimicrobial therapy in an immunocompetent host. However, antimicrobial therapy is warranted to treat enterocolitis in compromised hosts and in patients with septicemia or invasive infection, in which the mortality can be as high as 50%. Despite antibiotic susceptibility patterns varying among serogroups, the organism is usually susceptible in vitro to Aminoglycosides, Cotrimoxazole, Chloramphenicol, Tetracycline, third-generation Cephalosporins, and Fluoroquinolones but is resistant to Penicillin, Ampicillin and first-generation Cephalosporins (Bent and Young, 2010; EFSA and ECDC, 2016). The foods contaminated with *Yersinia enterocolitica* infection in humans are those of animal origin (Rahimi *et al*., 2014). Besides post-infection events, *Yersinia enterocolitica* can cause also primary cutaneous infections, endocarditis, pneumonia and other nosocomial infections, such as meningitis, septicemia, osteomyelitis, pharyngitis, and conjunctivitis (Bottone, 2015; Bonardi *et al*., 2016). Anzali Wetland is a large complex of freshwater lagoons with extensive reed beds and seasonally flooded meadows that have been recorded on the Ramsar Convention which is the only global environmental treaty that deals with a particular ecosystem. The Anzali wetland is a large complex environment of freshwater lagoons with extensive reed beds, shallow impoundments and seasonally flooded meadows. It is extremely important as a spawning and nursery ground for fish and as a breeding, staging, and wintering area for a wide variety of waterfowl. It is located in the northern part of Iran, along the coast of the Caspian Sea approximately at a north latitude between 37 25′ and 37 32′ and east longitude between 49 15′ and 49 36′. It has a catchment area of 3610 km^2 (Vesaili Naseh *et al.*, 2012; Salamatn *et al*., 2014; Sadeghi Pasvisheh *et al.*, 2021). This wetland is internationally well-known as a good place for passing through the larval stages and spawning many kinds of aquatics (Vesaili Naseh *et al*., 2012). Environmental conditions in the Anzali Wetland have been degraded due to the increased inflow of sewerage, wastewater,

solid waste from industry, agriculture, and urban areas, and sediment from the upper stream mountainous areas. The lagoon has becom smaller since the 1930s to less than a quarter of its former size, and its depth has decreased from 11 meters to less than 2.5 meters (Asadi, 2016; Sadeghi Pasvisheh *et al.,* 2021). The present study aimed to focus on the presence of *Yersinia enterocolitica* in fish, sampled from the Anzali wetland and antibiotic resistance evaluation of isolates.

Materials and methods

Sampling

Sampling was carried out for one year in four seasons from the autumn 2018 to the summer 2019 from four geographical directions: east, west, north, and south from different Anzali wetland levels by a simple random method. Fishing was limited in different areas of the wetland and all samples were collected with the help of local fisherfolks. A total number of 91 fish, Carp (n=27), Bream (n=14), Anchovy (n=23), Pike Perch (n=17), and Pike (n=10) were sampled from the body of these fish.

Microbiology tests

To isolate *Yersinia enterocolitica* from the studied samples, each fish body was cultured in the *Yersinia* Enrichment Broth medium (Merck, Germany) and incubated for 48 hours at 29°C. It was cultured linearly in Yersinia Selective Agar (Merck, Germany) and incubated at 37°C for 24 hours. After 24 hours, red colonies with a dark centers and translucent margins, 2-4 mm in diameter, were selected as suspect colonies to *Yersinia* and purified cultures were prepared on TSA (Merck, Germany) (Shakerian *et al.,* 2011). Subsequent microbial differentiation experiments were performed on suspected colonies extracted from the specific culture. Gram staining was then performed to observe small gram-negative bacilli. Biochemical tests were performed on suspected bacteria after confirmation at the staining stage.

DNA Extraction

PCR was performed for the definitive detection of *Yersinia enterocolitica.* Purification of DNA from bacterial colonies was achieved using a genomic DNA purification kit (Fermentas, Germany) according to the manufacturer's instructions. To specifically amplify the *Yersinia enterocolitica* 16S rRNA gene, a set of primers, Y1 (5'-AATACCGCATAACGTCTTCG-3)) and Y2 (5'-CTTCTTCTGCGAGTAACGTC-3)), was used (Thoerner *et al*.,2003; Shanmugapriya *et al.,* 2014).

Polymerase Chain Reaction (PCR)

PCR reaction in the volume of 50 µL including 5 µL PCR buffer10, 150 µmol dNTP mix, 2 mmol $Mgcl_2$, 1 µmol specific primer pairs, 1 the unit single DNA Taq polymerase enzyme, 1 µL from the DNA of each sample with temperature program included an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 94°C for 60 sec, annealing at 59°C 70 sec, and 72°C for 80 sec and a final extension at 72°C for 7 min, resulting in a PCR product of 330 bp (Wannet *et al.*, 2001; Thoerner *et al*., 2003; Shanmugapriya *et al.*, 2014). The PCR products were stained with 1% ethidium bromide solution and visualized under UV light after electrophoresis on 1.5% agarose gel (Fig. 1).

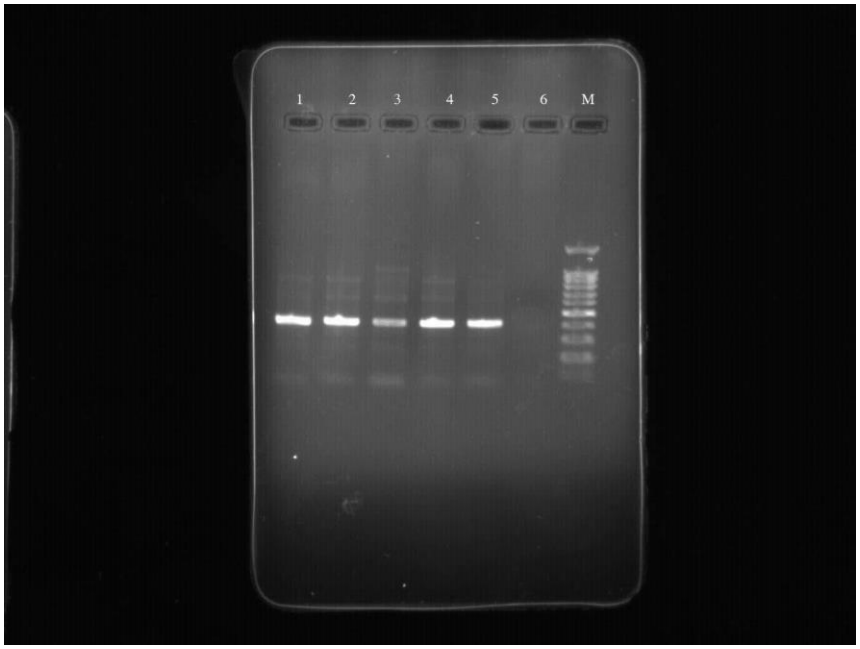

Figure1: Gel electrophoresis of PCR product of *Yersinia enterocolitica* Column M: 100-bp DNA marker, columns 1, 2, 3, 4 and 5 of the positive samples studied, column 6 of the negative samples studied.

Antimicrobial susceptibility testing

The antibiotic susceptibility of *Yersinia enterocolitica* isolates was determined with the Kirby-Bauer disc diffusion method as described by the National Committee for Clinical Laboratory Standards (NCCLS), and the zones of inhibition were measured and interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2016). The 10 antibiotic discs used were Kanamycin, Gentamicin, Tetracycline, Streptomycin, Ampicillin, Nalidixic acid, Ceftazidime, Cotrimoxazole, Azithromycin and Imipenem. The susceptibility of the isolates to the antibiotic was estimated due to the diameter of the growth zone around the disc (Fabrega and Vila, 2011) (Fig. 2).

Statistical Analysis

The data were analyzed using SPSS software version 24 using the chi-square test at cross-sectional levels, at a significance level of 0.05%.

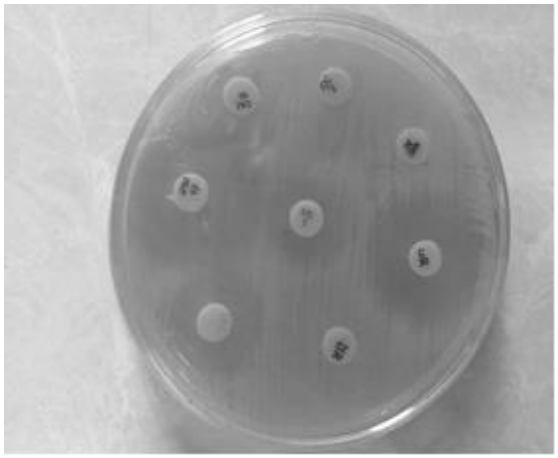

Figure 2: Sensitivity and resistance of the antibiotics study due to growth (sensitive) and lack of growth (resistant) to *Yersinia enterocolitica*.

Results

Results of the present study, which confirmed the culture method, show that from 91 fish samples, after biochemical analysis of positive samples, it was recognized that from a total of 91 samples, 20 (21.97 %) were positive for *Yersinia enterocolitica* (Tables 1 and 2).

Table 1: Biochemical characterization of *Yersinia enterocolitica* Isolated From fish meat samples in Anzali Wetland.

Substrate or Test	No. tested	No. positive	Positive (%)
Gram staining	91	0	0
Voges –Proskauer	91	71	78
Urease	91	44	48
Sorbitol	91	49	54
Ornithine decarboxylation	91	56	62
Citrate	91	39	43
DNase	91	39	43
Raffinose	91	43	47
Esculin hydrolysis	91	8	9
Salicin fermentation	91	10	11
Lysine Iron Agar (LIA)	91	51	56
H_2S	91	0	0

Table 2: Frequency of *Yersinia enterocolitica* isolated from fish meat caught in Anzali Wetland.

Type of fish	Number of samples	Culture Method	PCR Method
Crap	n = 27 (29.6%)	n = 8 (29.6%)	n = 6 (22.2%)
Bream	n = 14 (15.4 %)	n = 1 (7.14%)	n = 0 (0 %)
Anchovy	n = 23 (25.3%)	n = 4 (17.39%)	n = 3 (13.04%)
Pike Perch	n = 17 (18.7%)	n = 6 (35.29%)	n = 4 (23.5%)
Pike	n = 10 (11%)	n = 2 (20%)	n = 1 (10 %)
Total	n = 91 (100%)	n = 20 (21.97%)	n = 14 (15.4%)

After the PCR method, it was recognized that from a total of 91 samples, 14 (15.4%) were positive for *Yersinia enterocolitica* 16 rRNA gene (Table 2). Table 3 shows the distribution of *Yersinia enterocolitica* in fish samples in various seasons.

Table 3: Distribution of *Yersinia enterocolitica* in fish samples during various seasons.

Season	Fish Type	Number of samples	Positive samples for *Yersinia enterocolitica*
Autumn	Crap	12	2 (16.6%)
Autumn	Bream	8	0 (0%)
Autumn	Anchovy	8	0 (0%)
Autumn	Total	28	2 (7.14%)
Winter	Crap	6	2 (33.3%)
Winter	Bream	2	0 (0%)
Winter	Anchovy	4	1 (25%)
Winter	Total	12	3 (25%)
Spring	Crap	5	1 (20%)
Spring	Bream	2	0 (0%)
Spring	Anchovy	11	2 (18.18%)
Spring	Pike Perch	9	2 (22.2%)
Spring	Total	27	5 (18.51%)
Summer	Pike	10	1 (10%)
Summer	Crap	4	1 (25%)
Summer	Bream	2	0 (0%)
Summer	Pike Perch	8	2 (25%)
Summer	Total	24	4 (16.6%)

The winter had the highest (25%), while the autumn had the lowest (7.14%), and the spring (18.51%) also the summer (16.6%) frequency of *Yersinia enterocolitica* respectively (p>0.05). Table 2 displays the antibiotic susceptibility results of all 14 *Yersinia enterocolitica* isolated. The examined isolates were highly resistant to

Ampicillin (64.28%), also the most susceptible isolates were Imipenem (92.8%), Kanamycin, Gentamicin (85.7%), Ceftazidime (78.57%), respectively (Table 4). Statistical tests showed that there was no statistically significant difference between the various seasons (p>0.05) (Table 5), while there was a significant difference between fish species (p<0.05) (Table 6).

Table 4: Results of antibiotic susceptibility tests of *Yersinia enterocolitica* isolates.

Antibiotic	Susceptible	Resistant	Total
Kanamycin	12 (85.7 %)	2 (14.28 %)	14 (100%)
Gentamicin	12 (85.7 %)	2 (14.28 %)	14 (100%)
Tetracyclin	7 (50 %)	7 (50 %)	14 (100%)
Streptomycin	7 (50 %)	7 (50 %)	14 (100%)
Ampicillin	5 (35.7 %)	9 (64.28 %)	14 (100%)
Nalidixic acid	10 (71.4 %)	4 (28.57 %)	14 (100%)
Ceftazidime	11 (78.57 %)	3 (21.42 %)	14 (100%)
Cotrimoxazole	10 (71.4 %)	4 (28.57 %)	14 (100%)
Azithromycin	9 (74.28 %)	5 (35.71 %)	14 (100%)
Imipenem	13 (92.8 %)	1 (7.14 %)	14 (100%)

Table 5: Significance of samples in various seasons of *Yersinia enterocolitica.*

Seasons	Infected	Sig	Healthy
Spring	5 (18.5 %)		22 (81.5 %)
Summer	4 (16.7 %)		20 (83.3 %)
Autumn	2 (7.1 %)		26 (92.9 %)
Winter	3 (25 %)		9 (75 %)
P_1		0.86^{ns}	
P_2		0.21^{ns}	
P_3		0.64^{ns}	
P_4		0.28^{ns}	
P_5		0.55^{ns}	
P_6		0.1^{ns}	

P_1: Significance level of (Spring and Summer), P_2: Significance level of (Spring and Autumn), P_3: Significance level of (Spring and Winter), P_4: Significance level of (Summer and Autumn), P5: Significance level of (Summer and Winter), P6: Significance level of (Autumn and Winter), No significant differences were observed between the different seasons (p>0.05).

Table 6: Significance of samples in various fishes of *Yersinia enterocolitica*.

Type of fish	Infected	Sig	Healthy
Anchovy	3 (13 %)		20 (87 %)
Crap	6 (22.2 %)		21 (77.8 %)
Pike	1 (10 %)		9 (90 %)
Bream	0 (0 %)		14 (100 %)
Pike Perch	4 (23.5 %)		13 (76.5 %)
P_1		0.4^{ns}	
P_2		0.8^{ns}	
P_3		0.16^{ns}	
P_4		0.39^{ns}	
P_5		0.4^{ns}	
P_6		0.06^{ns}	
P_7		0.92^{ns}	
P_8		0.23^{ns}	
P_9		0.38^{ns}	
P_{10}		0.05 *	

P_1: Significance level of Anchovy and Carp, P_2: Significance level of Anchovy and Pike, P_3: Anchovy and Bream, P_4: Significance level of Anchovy and Pike Perch, P_5: Significance level of Carp and Pike, P_6: Significance level of Carp and Bream, P_7: Significance level of Carp and Pike Perch, P_8: Significance level of Pike and Bream, P_9: Significance level of Pike and Pike Perch, P_{10}: Significance level of Bream and Pike Perch, Significant differences in contamination between different fish (p<0.05).

Discussion

Yersinia enterocolitica is the cause of yersiniosis in humans and its symptoms range from mild diarrhea to mesenteric lymphadenitis that may lead to appendicitis (Garrity *et al* ., 2005). The severity of the clinical symptoms of this infection largely depends on a person's age, physical condition, and existence or non-existence of underlying disease, as well as the bacterial serotype (Petsios *et al*., 2016; Saraka *et al*., 2017). Observations of the high prevalence of the gastrointestinal diseases including victims of yersiniosis in many developing countries, including Bangladesh, Iraq, Iran, and Nigeria, prevalence of gastrointestinal diseases, including deaths from Yersiniosis. This suggests that underlying food safety problems are more prevalent in low- and middle-income countries (Schlundt, 2002; Kanan and Abdulla, 2009; Okwori *et al.*, 2009). Anzali Wetland is one of the most valuable natural ecosystems that has economic value in terms of biodiversity, ecotourism, and aquatic life. It also holds a special place in terms of being the major spawning center and nurseries for migrant and economic fish of the Caspian Sea such as Pike Perch, fish white and Bream (Feid *et al.*, 2015). The results of the present study in four seasons of different species of fish caught from Anzali wetland indicate15.4% of the samples were infected with *Yersinia enterocolitica*, also in the winter, the highest infection with *Yersinia enterocolitica* 25% and the lowest infection in the autumn was 7.14%. Contamination of food with antimicrobial-resistant bacteria is a threat to public health. The increased misuse or overuse of antimicrobials in animal production, veterinary medicine, and human therapy has resulted in increased resistance (Ozdemir and Arslan, 2015). In the present study, the most susceptible isolates, were imipenem (92.8%), Kanamycin and Gentamicin (85.7%), and Ceftazidime (78.57%). Cotrimoxazole and Nalidixic acid (71.4%) were the most resistant isolates to Ampicillin (64.28%), respectively. In the study of Wang, aiming investigating *Yersinia enterocolitica* as a cause of septicemia in Crucian carp, survey results in Twenty-eight crucian carps that were injected with strain G6029, died within a week, and the mortality was 93.3%, indicating the high pathogenic attribute of this strain. In addition, the strain ofG6029 was resistant to four antibiotics (Sulfafurazole, Furazolidone, Enrofloxacin and Norfloxacin), but it was susceptible to six antibiotics (Florfenicol, Vibramycin, Cefaloridine, Ciprofloxacin, Streptomycin, and Ampicillin) (Wang, 2016). The objectives of that study are similar to the ones of the current one and show the risk of *Yersinia enterocolitica* pathogenicity in fish. In the study, a total of 44 (20 chicken and 24 fish) samples were collected. About 55% of them (6 chicken and 18 fish) were found positive for the incidence of *Yersinia enterocolitica*. All the strains were resistant to amoxicillin, but sensitive to chloramphenicol, in general all exhibited multiple antibiotic resistance. 95.8%, 79%, 58% and 54% resistance to erythromycin, amikacin, imipenem and methicillin respectively (Shanmugapriya *et al.*, 2014). The results of this study show more infection with *Yersinia enterocolitica* compared to the present study and the

antibiotic resistance of the isolate is higher. A study in China aimed to estimate the extent of *Yersinia enterocolitica* contamination in frozen foods. Out of 455 samples collected between July 2011 and May 2014, 56 (12.3%) testes were positive for *Yersinia enterocolitica.* The 70 isolated strains were grouped into five clusters and one singleton based on their ERIC-PCR fingerprints, at a similarity coefficient of 70%. All strains were sensitive to ticarcillin but resistant to two or more antibiotics, and 48.6% of the strains were resistant to four to nine antibiotics. High resistance rates were observed for Ampicillin, Cephalothin, Trimethoprim/Sulfamethoxazole, Amoxicillin/Clavulanic acid, Nalidixic acid and Chloramphenicol (98.6%, 95.7%, 74.3%, 28.6%, 18.6%, and 12.9%, respectively) (Ye *et al*., 2016). The results of this study show less infection with *Yersinia enterocolitica* compared to the present study, but the antibiotic resistance of the isolate is higher. Another study in Poland showed that examination of 330 food samples (meat, white raw sausage, smoked meat, and cheeses) was conducted *Yersinia enterocolitica* prevalence was determined. Isolated *Yersinia enterocolitica* strains were bio typed *Yersinia enterocolitica* was found in 7 (2.1%) samples (Zaernowska and Chajecka-Wierzchowska, 2017). The results of this study show less infection with *Yersinia enterocolitica* compared to the present study. In the study, a total of 500 retail poultry meat samples from 4 provinces and 145 swine feces samples from 12 provinces in China were tested for *Yersinia enterocolitica* and 26 isolates were obtained for further bio-serotyping, testing with antimicrobial susceptibility testing to a panel of antimicrobial compounds, and genetic characterization based on the whole genome sequencing. A higher prevalence (4.8%) of *Yersinia enterocolitica* contamination in retail poultry meat than that in swine feces (2.76%) was observed. No difference in bio-serotypes, multilocus sequence typing (MLST) and virulence gene distribution between swine and poultry origins were found. All isolates were resistant to ampicillin, amoxicillin/clavulanic acid, and cefazolin and were multi-drug resistant (MDR). The most predominant drug-resistance profile was AMP-CFZ-AMC-FOX (42.31%). A pathogenic isolate with bio-serotype 3/O:3 and ST135 was cultured from retail fresh chicken meat for the first time in China (Peng *et al*., 2018). Less infection with *Yersinia enterocolitica* compared to the present study, but the antibiotic resistance of the isolate is higher. In the previous study, the contamination rate of *Yersinia enterocolitica* in Anzali wetland water was 41% in the specific culture method and 35.7% in PCR method (Abolhasani *et al.*,2020). The infection of the Anzali wetland water with *Yersinia enterocolitica* as a substrate for fish and its transmission through the food cycle shows the risk of pathogenicity.

Conclusions

This study reports that the incidence of *Yersinia enterocolitica* was higher in Carp and Pikle Perch than other fish, probably because both species live on the floor of the wetland, omnivorous. However, Pikle Perch species are more carnivorous and live in shallow areas and rivers, which may be

the cause of greater contamination of these fish than other species of fish. Also, in the winter, the rate of pollution was higher than in the spring, the autumn and the summer, which may be due to the cold tolerance of *Yersinia enterocolitica*, and the lowest level of pollution was in the autumn. The results were indicative of the potential dangers of *Yersinia enterocolitica* pathogenicity, especially among immunocompromised and susceptible individuals. Environmental conditions in the Anzali wetland have been degraded due to the increased inflow of sewerage, wastewater, solid waste discharged byindustries, agriculture, and urban areas, and sediment from the upper stream mountainous area. Further studies on the pathogenicity of *Yersinia enterocolitica* in fish in Anzali Wetland and preventing sewage from entering the wetland are suggested.

Conflicts of interest

The authors declare no conflict of interest.

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