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Prevalence of *Vibrio parahaemolyticus* in seabass (*Dicentrarchus Labrax*) and seabream (*Sparus aurata*) and Detection of Streptomycin-resistant Strains

Adel M. El-Gamal[1*] and **Engy F. EL-Bahi**[2]

[1]*Bacteriology unit. Animal Health Research Institute, Kafr El-Sheikh branch, Agriculture research center (ARC), Egypt.*
[2]*Food hygiene unit. Animal Health Research Institute, Kafr El-Sheikh branch, Agriculture research center (ARC), Egypt.*

**Corresponding author's Email:* adelelgamal5544@yahoo.com; ORCID: 0000-0002-7697-929X

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ABSTRACT

Vibrio species are the most common and serious pathogens in fish and shellfish marine aquaculture worldwide. The present study aimed to determine the prevalence of *Vibrio* spp. in seabass and seabream in fish markets, especially streptomycin-resistant strains that have great public health importance. A total of 30 seabass (*Dicentrarchus Labrax*) and 30 seabream (*Sparus aurata*) were purchased from fish markets at Kafr El Sheikh Governorate and subjected to bacteriological examination. The PCR assay was used for the detection of virulence genes (*tdh* and *trh*), aminoglycoside resistance gene (*aadA1)*, and *toxR* gene. The results indicated that the total prevalence of *Vibrio* spp. was 26.66%, including *V. parahaemolyticus* (8.3%), *V. alginolyticus* (8.3%), *V. mimicus* (3.3%), *V. harveyi* (5%) and *V. vulnificus* (1.6%). The *toxR*, *trh*, and *aadA1* genes were found in all *V. parahaemolyticus* isolates while *tdh* gene was found in 80% of isolates. Antimicrobial sensitivity test of *V. parahaemolyticus* isolates showed sensitivity to ciprofloxacin, norfloxacin, cefotaxime, and chloramphenicol. *Vibrio parahaemolyticus* isolates were resistant to ampicillin, erythromycin, streptomycin, and gentamycin. The present results indicated that good hygienic measures should be taken to avoid infection with *Vibrio* species, especially *V. parahaemolyticus* that can pose a great risk to human health.

Keywords: Antibiotic resistance, Seabass, Seabream, Streptomycin, *Vibrio parahaemolyticus*.

INTRODUCTION

Vibrio genus contains Gram-negative, halophilic, rod-shaped, non-spore forming, oxidase-positive bacteria, which widespread in the coastal and estuarine environments (Austin and Austin, 2007). *Vibrio parahaemolyticus* is the most recorded pathogenic species of *Vibrio* genus and affects persons who consume improperly cooked or raw seafood **(**Raissy et al., 2015). This foodborne bacteria is reported as the main cause of seafood-borne illness in Egypt and many other countries around the world such as United States, Malaysia, Thailand, Korea, China, and Japan (Yoon et al., 2008; Iwahori et al., 2010; Abdel-Azeem et al., 2016**)**. Infection with *V. parahaemolyticus* may cause acute human gastroenteritis, the major symptoms of which are headache, diarrhea, abdominal pain, and in some cases, septicemia (Broberg et al., 2011; Wang et al., 2015; Su and Liu, 2017). In coastal areas of the world, like Japan, *V. parahaemolyticus* has been regularly recognized as the main cause of sporadic cases of gastroenteritis (Qadri et al., 2005; Wang et al., 2017). In China, about 322 gastroenteritis outbreaks due to *V. parahaemolyticus* infection were reported from 2003 to 2008 (Wu et al., 2014). Multiplication of *V. parahaemolyticus* is related to water temperature and season (Deepanjali et al., 2005; Angela et al., 2006), with the highest prevalence in summer due to the higher salinity of water than other seasons (Zulkifli et al., 2009).

The pathogenicity of bacteria depends mainly on some virulence factors and virulence genes, which act together as major orchestrators. The most virulence genes leading to pathogenicity of *V. parahaemolyticus* are hemolysin genes (*tdh* and *trh*) (Hiyoshi et al., 2010). Molecular epidemiological studies demonstrated a clear relation between the hemolysin genes and disease-causing ability of *V. parahaemolyticus* (Kishishita et al., 1992; DePaola et al., 2003; Vongxay et al., 2008; Chao et al., 2009; Han et al., 2015; Hasrimi et al., 2018**)**. These two genes were recorded in the most isolates from clinical cases of *V. parahaemolyticus* infections **(**Bej et al., 1999; Rojas et al., 2011). The *tdh* and *trh* genes encode virulence factors of thermostable direct hemolysin (TDH), and TDH-related hemolysin (TRH), respectively, which are involved in important pathogenic activities, such as enterotoxicity, hemolytic activity, cytotoxicity and cardiotoxicity (Shirai et al., 1990; Osawa et al., 1996).

The *toxR* gene is a pandemic marker gene for all *V. parahaemolyticus* strains either pathogenic or nonpathogenic one, and it was recorded in some other *Vibrio* species (Kim et al., 1999). The sequence of *toxR* gene can be used for molecular identification of *V. parahaemolyticus* (Yung et al., 1999; Hubbard et al., 2016). The *aadA1* and *aadA2* encode

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aminoglycoside adenyl transferase and confer resistance to streptomycin, already are detected in *Vibrio* species isolates (Dalsgaard et al., 2001).

Cooking and frying of marine fish reduce the count of *V. parahaemolyticus.* After cooking (in oven 120 ºC for 35 min), the percentage reduction in total count of *V. parahaemolyticus* was 98.2%, while after frying for 10 min at 190 °C, *V. parahaemolyticus* was completely destroyed and the percentage reduction was 100% (Saad et al., 2015); or even boiling at 64 °C for more than 90 seconds can kill *V. parahaemolyticus* (ICMSF, 1996). The bacteria will be removed by using high cooking temperature, although the toxin might remain in the foodstuff depending on the processing conditions (Raissy et al., 2015). The current study aimed to determine the prevalence of *Vibrio* spp., especially streptomycin-resistant strains, in seabass and seabream in fish markets of Kafr El Sheikh Governorate, Egypt.

MATERIALS AND METHODS

Samples collection

Thirty seabass and 30 seabream with a weight range of 100-250 g were purchased from fish markets at Kafr El Sheikh Governorate from February to August 2019. All samples were transferred in ice box to Animal Health Research Institute, Kafr El Sheikh laboratory, Egypt.

Bacteriological examination

Bacteriological examinations were done according to ISO/ TS 21872-1 (2007) and ISO/ TS 21872-2 (2007).

Samples preparation

After skin sterilization with alcohol, the muscles above the lateral line were removed, 25 g of each fish sample were mixed with 225 ml of alkaline saline peptone water (APW, pH 8.6) in a Stomacher bag. After that, these mixtures were incubated at 37 °C for 8-16 hours.

Isolation of *Vibrio* species

After the incubation period, the upper layer of the alkaline saline peptone water (APW) enrichment broth was inculcated on Thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Oxoid, UK), and then these plates were incubated at 37 °C for another 18-24 hours. After that, growing colonies were used for further screening tests including Gram staining, oxidase and catalase tests.

Biochemical identification

Suspected colonies of *Vibrio* spp. on TCBS media and positive oxidase test were subjected to further identification by Microbact GNB kit (Oxoid, UK).

Polymerase chain action

Suspected isolates of the *V. parahaemolyticus* were examined by using PCR for the detection of virulence genes (*tdh* and *trh*), *toxR* gene, and *aadA1* gene. DNA extraction were performed according to the manufacturer's recommendations by using the QIA amp DNA Mini kit (Qiagene, Germany, GmbH). Oligonucleotide primers were supplied from Metabion (Germany). The primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan) using an Applied Biosystem 2720 thermal cycler. Primers used and PCR conditions are presented in Table 1. The products of PCR were separated by electrophoresis on 1% Agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5 V/cm. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra).

Antimicrobial susceptibility test

Antimicrobial disk susceptibility test were performed as described by the Clinical and Laboratory Standards Institute (CLSI, 2012).

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions.

Target genes	Primers Sequence (5'-3')		Amplified segment (base pair)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
toxR	F	*GTCTTCTGACGCAATCGTTG*	368	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Kim et al., 1999
	R	*ATACGAGTGGTTGCTGTCATG*							
aadA1	F	*TATCAGAGGTAGTTGGCGTCAT*	484	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Randall et al. 2004
	R	*GTTCCATAGCGTTAAGGTTTCATT*							
trh	F	*GGCTCAAAATGGTTAAGCG*	250	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Mustapha et al., 2013
	R	*CATTTCCGCTCTCATATGC*							
tdh	F	CCATCTGTCCCTTTTCCTGC	373	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 40 sec.	72˚C 7 min.	
	R	*CCAAATACATTTTACTTGG*							

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RESULTS AND DISSCUSION

Vibrio spp. commonly inhabit the marine environments and can be found in the fresh water (Sujeewa et al., 2009). Seafood may be a vehicle for most of the bacterial pathogens such as *Vibrio* spp. (Huss, 1997). Various outbreaks of bacterial disease associated with seafood consumption have been reported (Friesema et al., 2012). Recently, *V. parahaemolyticus* recoded as an important species causing seafood infection associated with gastroenteritis illness in humans.

Table 2 shows that the total incidence of *Vibrio* spp. isolated from the examined seabass and seabream samples is 26.66% (16 out of 60 samples). Raissy et al. (2015) and Azwai1 et al. (2016) recorded nearly similar results (22% and 22.9%, respectively). however, the result of present study is lower than that recorded by Pal and Das (2010), Saad et al. (2015), Abdel-Azeem et al. (2016), Fri et al. (2017), and Hemmat et al. (2018).These differences may be due to difference in the type of examined fish, difference in the method of bacterial isolation, or difference in the hygienic state of fish sources. Additionally, the difference in results can be attributed to difference in season of sampling, as *Vibrio* spp. has been reported to have higher concentrations in summer seasons due to higher water salinity levels than other seasons (Zulkifli et al., 2009). As presented in table 2, several *Vibrio* strains were isolated from examined seabass and seabream, including *V. parahaemolyticus* (8.3%), *V. alginolyticus* (8.3%), *V. mimicus* (3.3%), *V. harveyi* (5%) and *V. vulnificus* (1.6%). *Vibrio cholera* was not detected in the studied samples. The examined seabass fish were more infected with *V. parahaemolyticus* than the examined seabream fish which may be due to the hygienic state of each fish source.

Similarly, Saad et al. (2015) isolated *V. parahaemolyticus* (10%), *V. fluvialis, V. vulnificus, V. alginolyticus , V. mimicus,* and *V. damsel* from *Tilapia nilotica* and *Mugil Cephalus*. Hemmat et al. (2018) isolated *V. parahaemolyticus* (12%), *V. mimicus, V. alginolyticus*, *V. cholera*, *V. vulnificus,* and *V. fluvialis* from *Oreochromis niloticus*, *Mugil Cephalus*, shrimp and crab. Raissy et al. (2015) isolated *V. harveyi* that was the most frequent species isolated, followed by *V. parahaemolyticus* (3.5%), *V. mimicus, V. vulnificus,* and *V. alginolyticus* from some marine fish and shrimps. Fri et al. (2017) isolated *V. fluvialis, Vibrio vulnificus,* and *V. parahaemolyticus* (5.45%) from dusky kop fish and sea water. Pal and Das (2010) isolated *Vibrio parahaemolyticus* with a high prevalence (35%) from shrimp, prawn, bhetki, pamfret and hilsa. According to the Egyptian Organization for Standardization and Quality Control (EOSQC, 2005), any seafood products must be free from *V. parahaemolyticus*.

As shown in table 3 and figure 1, all examined *Vibrio parahaemolyticus* isolates were positive for *toxR* gene. This result support finding of Yung et al. (1999); Pal and Das (2010), who reported that *toxR*-targeted PCR protocol can be used for *V. parahaemolyticus* detection. Also, all examined *Vibrio parahaemolyticus* isolates were positive for *aadA1* gene (Figure 2). Taviani et al. (2008) stated that *aadA1* gene is responsible for antibiotic resistance against aminoglycoside group including streptomycin in *Vibrio* spp. isolates from shellfish and other marine fish.

Pathogenicity of *V. parahaemolyticus* is conferred either by *tdh*, and/or *trh* (Yamaichi et al., 1999**)**. As shown in table 3, all examined *V. parahaemolyticus* isolates were positive for *trh* gene (figure 3), and 80% were positive for *tdh* gene (Figure 4). The results did not match with that reported by Rojas et al. (2011) who detected *tdh* gene in 10.5% of *V. parahaemolyticus* isolates, while *trh* gene was not found. Also, Pal and Das (2010) recorded *tdh* gene in 35% of *V. parahaemolyticus* isolated from fish samples while *trh* gene was found only in 1.7% of *V. parahaemolyticus* isolates. Wang et al. (2017) recorded the virulence genes; *tdh* and *trh* with 87.9% and 3.7% of examined *V. parahaemolyticus* strains, respectively. Fri et al. (2017) recorded *trh* gene as 9.46% in examined *V. parahaemolyticus* strains, while Wong et al. (2000) recorded only one *V. parahaemolyticus* isolate (1.4%) harboring *trh* gene, but did not detect *tdh* gene among the examined *V. parahaemolyticus* isolates.

Antimicrobial susceptibility test showed that *V. parahaemolyticus* isolates were sensitive to ciprofloxacin, norfloxacin, cefotaxime, and chloramphenicol while they were resistant to ampicillin, erythromycin, streptomycin, and gentamycin (Table 4). These results indicate that the examined strains were resistant to most members of the aminoglycoside group, which may be due to the fact that *aadA1* gene was detected in all examined *V. parahaemolyticus* isolates. This result is nearly similar to that recorded by Rojas et al. (2011), who reported that *V. parahaemolyticus* had resistance to streptomycin and ampicillin with intermediate susceptibility to gentamicin.

Table 2. Prevalence of *Vibrio* species in examined seabass and seabream fish, Egypt.

***Vibrio* spp.**	**Number of positive samples**		
	Seabass (n=30)	**Seabream (n=30)**	**Total (%)**
V. parahaemolyticus	1	4	5 (8.3)
V. alginolyticus	3	2	5 (8.3)
V. mimicus	0	2	2 (3.3)
V. harveyi	1	2	3 (5)
V. vulnificus	1	0	1 (1.6)
Total	6	10	16(26.6)

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Table 3. Distribution of virulence genes among examined isolates of *Vibrio parahaemolyticus* isolated from seabass and seabream fish.

Sample No.	*toxR*	*tdh*	*trh*	*aadA1*
1	+	+	+	+
2	+	-	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+

Table 4. Results of agar disc diffusion test of *Vibrio parahaemolyticus* isolated from marine fish

Antibiotic	Disc symbol & concentration (µg/disc)	Result
Norfloxacin	Nor (10)	S
Erythromycin	E (15)	R
Ampicillin	AMP (10)	R
Amoxicillin + clavulinic acid	AMC (30)	S
Cefotaxime	CTX(30)	S
Doxycycline	DO (30)	R
Streptomycin	S(10)	R
Sulpamethazol + Trimethoprim	SXT(25)	R
Chloramphenicol	C (30)	S
Gentamycin	CN(10)	R
Ciprofloxacin	Cip (5)	S

S: Sensitive R: Resistant

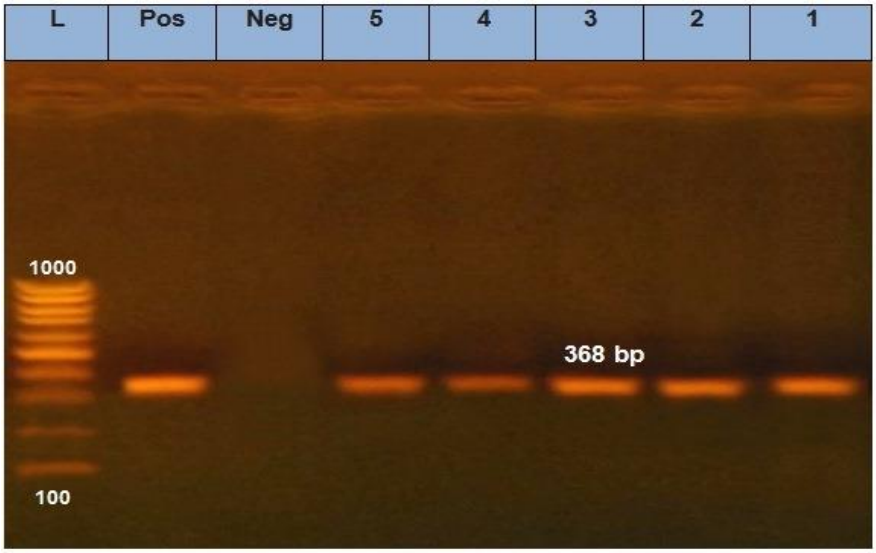

Figure 1. Agarose gel electrophoresis of PCR amplification of *toxR* gene (368 bp) of *Vibrio parahaemolyticus.* Lane L: 100-600 bp DNA Ladder. Neg.: Negative control. Pos.: Positive control. Lane 1-5: Positive samples.

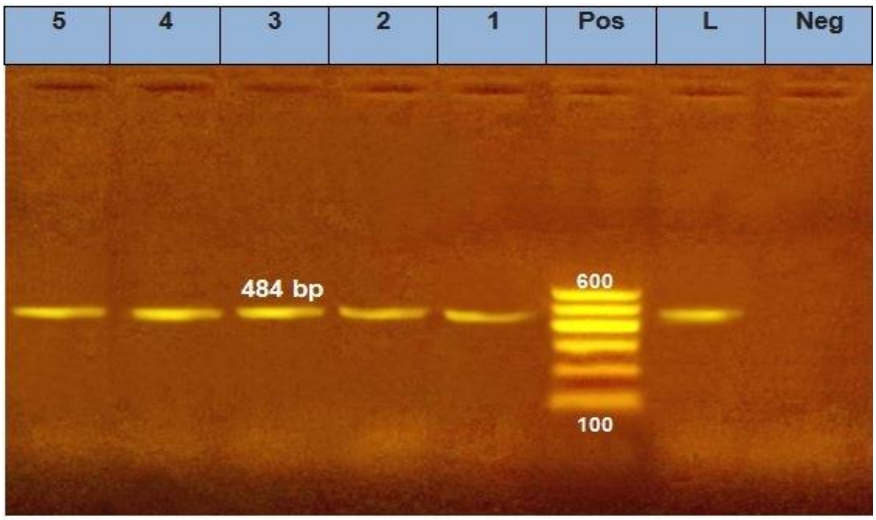

Figure 2. Agarose gel electrophoresis of PCR amplification of *aadA1 gene* (484 bp) of *Vibrio parahaemolyticus.* Lane L: 100-600 bp DNA Ladder. Neg.: Negative control. Pos.: Positive control. Lane 1-5: Positive samples.

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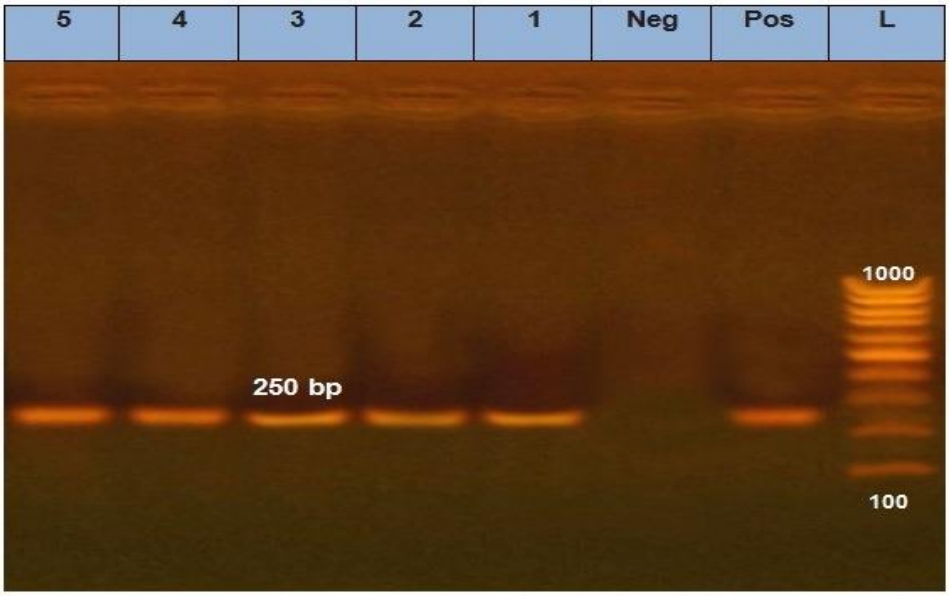

Figure 3. Agarose gel electrophoresis of PCR amplification of *trh* gene (250 bp) of *Vibrio parahaemolyticus.* Lane L: 100-600 bp DNA Ladder. Neg.: Negative control. Pos.: Positive control. Lane 1-5: Positive samples.

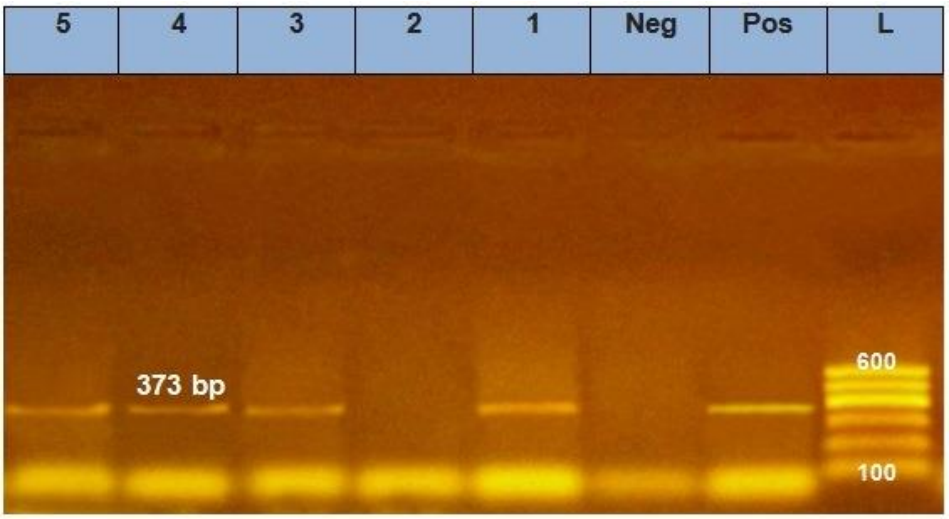

Figure 4. Agarose gel electrophoresis of PCR amplification of *tdh* gene (373bp) of *Vibrio parahaemolyticus*. Lane L: 100-600 bp DNA Ladder. Neg.: Negative control. Pos.: Positive control. Lane 1,3,4 and 5: positive samples, Lane 2: negative sample.

CONCLUSION

Vibrio spp. especially *V. parahaemolyticus*, *V. alginolyticus*, *V. mimicus*, and *V. vulnificus* are commonly isolated from seabass and seabream fish, which affects persons who consume improperly cooked or raw seafood. Most of these bacteria have antibiotic resistance genes that pose a great risk to human health; therefore, good hygienic measures should apply to avoid such infections.

DECLARATIONS

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Authors' contributions

All authors participated equally in study design, data collection, data analysis, writing, and approving the final manuscript**.**

Competing interests

The authors declare that they have no competing interests.

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