THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Study on *Streptococcus parauberis* serotypes isolated from farmed olive flounder (*Paralichthys olivaceus*) in Jeju Island from 2019 to 2023

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Study on *Streptococcus parauberis* serotypes isolated from farmed olive flounder (*Paralichthys olivaceus*) in Jeju Island from 2019 to 2023

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Abstract

S. parauberis, causing streptococcosis, is a significant bacterial disease affecting olive flounder production. Although studies on *S. parauberis* serotypes are ongoing, there has been no research on the serotypes isolated from cultured olive flounder in Jeju Island since 2020. Therefore, this study investigates the *S. parauberis* serotypes isolated from cultured olive flounder in Jeju from 2019 to 2023. From March 2019 to August 2023, we analyzed 382 *S. parauberis* isolates obtained from infected olive flounder in Jeju Island farms. In 2019, 155 isolates were collected, followed by 80 in 2020, 30 in 2021, 32 in 2022, and 85 in 2023. All 382 isolates were categorized into three serotypes. Among them, subserotype Ia was the most prevalent, constituting 73% of the isolates (279 isolates). Subserotype Ib/Ic accounted for 16% (61 isolates), while serotype II made up 11% (42 isolates). Subserotype Ia consistently exhibited a high detection rate each year, while subserotype Ib/Ic showed a decreasing trend. Conversely, serotype II demonstrated an increasing trend. The API 20 STREP kit was employed to assess biochemical distinctions based on serotypes, but no such differences were identified.

Antibiotic susceptibility testing revealed high resistance to quinolones, sulfonamides, and aminoglycosides. The detection of antibiotic resistance genes varied according to serotypes, with *tet*(S) and *erm*(B) detected in subserotype Ia and *tet*(M) confirmed in serotype II. Antigen vaccines containing *S. parauberis* serotypes include all subserotype Ib/Ic and three types that include subserotype Ia. Throughout the experimental period, monitoring the serotypes revealed a consistent high detection rate for subserotype Ia, along with an increasing trend in the detection rate of serotype II. As the prevalence of *S. parauberis* serotypes may change over time, awareness of the possibility that the current vaccine's antigen serotypes may not match the latest prevalent serotypes is crucial. Therefore, reliance on vaccines based on serotypes and serotype patterns before vaccination is considered important. For these reasons, this study serves as a reference for future *S. parauberis* serotype analyses.

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1. Introduction

In the late 1980s, land-based aquaculture systems, focusing on cultivating olive flounder (*Paralichthys olivaceus*), were initiated in Jeju Island. With advancements in aquaculture technology, the production of olive flounder, which was approximately 4,000 tons in 1993, has surged to 45,801 tons, constituting 50.6% of the total farmed fish production in South Korea, establishing itself as a major species in aquaculture. In 2022, out of the total domestic farmed fish production of 90,545 tons, farmed olive flounder alone reached 45,801 tons, with 49.1% of this production occurring in Jeju Island (Ok et al., 2006; KOSIS, 2022). However, the pursuit of increased production through high-density farming has led to the emergence of diseases. The lack of effective aquaculture management and environmental degradation has adversely impacted olive flounder production (Oh et al., 1998; Eh et al., 2011).

Diseases causing economic losses in olive flounder have been reported as bacterial diseases, parasitic diseases, and viral diseases (Shim et al., 2019). Among bacterial diseases that commonly occur in olive flounder, streptococcosis, edwardsiellosis, and vibriosis have been documented (Oh et al., 2006; Cho et al., 2007; Kwon et al., 2012).

The causative agents for streptococcosis, which infect farmed fish and induce diseases, include *Streptococcus iniae*, *S. parauberis*, *S. agalactiae*, *S. dysgalactiae*, and *Lactococcus garvieae*. Streptococcosis is known to occur in freshwater and saltwater fish (Kusuda et al., 1978; Baeck et al., 2006; Austin and Austin, 2007; Pradeep, 2016; Mishra et al., 2018), and was first reported in Japan in 1957. It was first reported in rainbow trout (*Oncorhynchus mykiss*) farm in Japan in 1957 (Hoshina et al., 1958). Subsequent cases were observed in tilapia (*Tilapia nilotica*) and ayu (*Plecoglossus altivelis*) farms in Japan in 1981 (Kitao et al., 1981), and in 1982, infections were reported in the mediterranean crab (*Carcinus mediterraneus*) (Pappalardo and Boemare, 1982).

In 1993, infection was reported at a turbot (*Scophthalmus maximus*) farm in Spain (Toranzo et al., 1994). There is evidence of *S. parauberis* distribution in the coastal waters of Jeju Island (Roh et al., 2018). As a result, streptococcosis is reported to infect a variety of fish species and is widely distributed across different regions.

Major symptoms of streptococcosis include darkening of body color, abdominal distension, ascitic fluid in the peritoneal cavity, petechial hemorrhaging, hemorrhaging on the non-ocular side, hemorrhagic septicemia, exophthalmia, and meningitis, and an splenomegaly (Chang et al., 1996; Jeong et al., 2006; Baeck et al., 2006; Kim et al., 2006; Nho et al., 2009; Harvie et al., 2013). The streptococcosis that occurs in olive flounder produced domestically is known as *S. iniae*, *S. parauberis*, and *L. garvieae*. (Kim et al., 2018; Lee et al., 2020). However, *S. parauberis* is more frequently detected than other streptococcosis agents (Woo et al., 2014). The first infection of *S. parauberis* was reported in farmed olive flounder on Jeju Island in 2005 (Baeck et al., 2006), and since the early 2010s, *S. parauberis* has been reported as the primary causative agent of streptococcosis predominantly occurring in domestically farmed olive flounder (Jeong et al., 2006; Baeck et al., 2006; Kim et al., 2020).

S. parauberis primarily targets the heart and brain, with representative symptoms including pericarditis and meningitis (Choi et al., 2009; Won et al., 2010; Kim et al., 2017). *S. parauberis* is a non-motile, catalase-negative, gram-positive bacterium (Toranzo et al., 1994). Initially identified as *S. uberis* genotype II due to its similarities in biochemical and serological characteristics with the causative agent of clinical mastitis in dairy cows, *S. uberis*, it was later distinguished as a separate bacterium, *S. parauberis*, through 16S ribosomal RNA sequence analysis (Williams et al., 1990).

Since the differentiation of *S. parauberis* serotypes, various serotype studies have been conducted to date. In 2009, an agglutination assay with rabbit

antiserum confirmed the division of *S. parauberis* into serotype I and serotype II (Kanai et al., 2009). The capsule layer on the bacterial cell surface, known for providing resistance against the host's innate and acquired immunity, has been reported to influence the pathogenicity of bacteria, with differences in thickness reflecting varying pathogenic outcomes (Johnson et al., 1992; Wai et al., 1998; Hwang et al., 2008; Han et al., 2011). For this reason, it was confirmed that *S. parauberis* serotype I had a thicker capsule layer than serotype II, indicating a difference in pathogenicity between the two serotypes (Han et al., 2011). In 2015, it was confirmed that serotype I can be further divided into three subserotypes, Ia, Ib, and Ic (Kanai et al., 2015). Through an agglutination assay with rabbit antiserum, strains that did not agglutinate to serotype I and serotype II were identified, and based on the degree of agglutination, the serotypes were divided into Ia, Ib, and Ic. Using pulsed-field gel electrophoresis (PFGE), it was reported that serotype I can be further divided into two subserotypes, Ia and Ib/Ic. In 2015, primers specific to the polysaccharide polymerase gene (wzy) of *S. parauberis* were designed for subserotype Ia, subserotype Ib/Ic, and serotype II, and through multiplex polymerase chain reaction (PCR), it was confirmed that three serotypes could be identified (Tu et al., 2015). In 2020, serotype III of *S. parauberis* was isolated from turbot (*Scophthalmus maximus*) in Spain (Torres-Corral et al., 2020). Also, the diversity in serotypes of *S. parauberis* is confirmed to be caused by capsular polysaccharide (CPS) (Kanai et al., 2009; Kanai et al., 2015; Torres-Corral et al., 2020).

According to previous research three serotypes of *S. parauberis* were identified of olive flounder cultured in Jeju Island, and the distribution of *S. parauberis* serotypes varied over specific periods (Kim et al., 2020). However, no research on serotype monitoring of *S. parauberis* has been conducted in Jeju Island since 2020.

In the domestic veterinary pharmaceutical sector, approximately 20 types of

drugs are currently registered and in use, with tetracycline antibiotics constituting around 70% of them. Following that, there are β-lactam and quinolone antibiotics, and the remaining include a small amount of macrolide and sulfonamide antibiotics (Jo et al., 2015). In 2018, the total amount of antibiotics used in cultured olive flounder in the Jeju Island was confirmed to be 322 tons. Particularly, tetracycline antibiotics and penicillin antibiotics were the most widely used, followed by aminoglycoside, penicol, quinolone, sepharoseporin, lincosamide, and macrolide antibiotics (Jeong, 2021). The tetracycline antibiotics, including oxytetracycline, doxycycline, and tetracycline, are widely used in aquaculture to minimize losses caused by bacterial diseases. Additionally, antibiotics such as ampicillin and amoxicillin from the β-lactam antibiotics, flumequine, oxolinic acid, and nalidixic from the quinolone antibiotics, as well as erythromycin from the macrolide antibiotics and clindamycin from the lincosamide antibiotics, are employed in the treatment of vibriosis and streptococcosis infections in olive flounder. Due to the lack of clear regulations on antibiotic use in the country, antibiotics are administered freely without undergoing susceptibility testing when diseases occur. Consequently, this has led to an increase in resistance levels to conventional antibiotics and a decline in treatment efficacy (Kim et al., 2022).

Tetracycline antibiotics inhibit bacterial cell wall synthesis, thereby suppressing the normal functioning of bacteria (Schnappinger et al., 1996). However, the increasing prevalence of resistant bacteria poses challenges in the treatment of infections (Lee et al., 2017). Resistance to tetracycline primarily occurs due to genes such as *tet*, which are involved in drug efflux pump, ribosomal protection or enzymatic activation modification (Levy et al., 1999; Chopra and Roberts, 2001; Giovanetti et al., 2003). Macrolide antibiotics inhibit RNA-dependent protein synthesis by reversibly binding to the 50S ribosome of susceptible bacteria (Jeong, 2003). Resistance to erythromycin, a type of macrolide, is mainly associated with *erm* and *mef* gene families,

which alter the ribosomal target through methylation or mutation, leading to resistance (Sapkota et al., 2006).

In this study, the focus was on collecting serotype data of *S. parauberis* from olive flounder suspected of infection in Jeju Island aquaculture facilities spanning from 2019 to 2023. Serotype monitoring, biochemical analysis, susceptibility testing to commonly used antibiotics in olive flounder, and detection experiments for antibiotic resistance genes were conducted. The objective is to lay the groundwork for the development of an olive flounder *S. parauberis* vaccine and serotype monitoring.

2. Materials and methods

2.1. Isolation of bacterial strains

Experimental strains were isolated from diseased olive flounder in aquaculture facilities located in Jeju-si (Hangyeong, Jocheon, Gujwa) and Seogwipo-si (Daejeong, Namwon, Pyoseon, Seongsan) from March 2019 to August 2023 (Fig. 1). For strain identification, internal organs of olive flounder was streaked on tryptic soy agar (TSA, Difco, USA) and brain heart infusion agar (BHIA, Difco, USA), as well as selective media including thiosulfate citrate bile salts sucrose (TCBS) agar (Difco, USA) and salmonella shigella (SS) agar (MB cell, Korea). The cultures were then incubated at 28°C for 24 hours to confirm colony formation and morphology. To distinguish *Streptococcus* spp. strains, the criterion was the formation of small white colonies on TSA plates. *S. parauberis*, strains with pure culture isolation were cultured in tryptic soy broth (TSB, Difco, USA) supplemented with 2% NaCl at 28°C for 24 hours Experimental strains were preserved by adding 20% glycerol (Sigma, USA) and stored at -80°C.

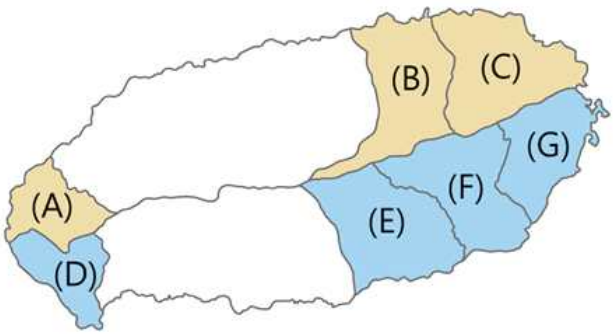

Fig. 1. Location of the olive flounder farms station in this study. Yellow area, olive flounder farms located in jeju-si; bule area, olive flounder farms located in seogwipo-si. (A) Hangyeong; (B) Jocheon; (C) Gujwa; (D) Daejeong; (E) Namwon; (F) Pyoseon; (G) Seongsan.

2.2. DNA extraction

For the DNA extraction of *S. parauberis*, strains with pure culture isolation were cultured in TSB supplemented with 2% NaCl at 28°C for 24 hours. Genomic DNA was extracted using the HiGene^{TM} Genomic DNA Prep Kit (Biofact, Korea).

In a 1.7 ㎖ microtube, 1.5 ㎖ of bacterial culture in TSB was centrifuged at 12,000 rpm for 5 minutes, and the supernatant was discarded. The pellet was resuspended in 300 ㎕ of cell re-suspension solution, and 2 ㎕ of lysozyme was added, followed by a 1 hour incubation at 37°C. After incubation, centrifugation at 13,000 rpm for 1 minute removed the supernatant. To the pellet, 300 ㎕ of cell lysis solution was added, and after resuspension, 1.5 ㎕ of RNase A was added, followed by a 30 minute incubation at 37°C. Afterward, the sample was cooled for 5 minutes at room temperature. Protein precipitation solution (100 ㎕) was added, strongly vortexed, and centrifuged at 13,000 rpm for 5 minutes. The supernatant (500 ㎕) was transferred to a new 1.7 ㎖ microtube, where 500 ㎕ of 100% isopropanol was added. After 50 inverting, centrifugation at 13,000 rpm for 1 minute removed the supernatant. Ethanol (80%, 500 ㎕) was added, followed by 50 inverting and centrifugation at 13,000 rpm for 1 minute. After discarding the supernatant, the sample was air-dried at room temperature for 10~15 minutes and then hydrated with 50 ㎕ of DNA hydration solution. Extracted DNA was stored at -80°C until further use (Lee, 2018a).

2.3. Polymerase chain reaction (PCR) analysis

2.3.1. Identification of *S. parauberis*

For the identification of *S. parauberis*, PCR was conducted using primer sets proposed by Woo et al. (2006) and is shown in Table 1. In a 0.2 ㎖ microtube, a final PCR mixture of 20 ㎕ was prepared by adding 1 μM of each primer, 2.5 mM of each dNTP (COSMO genetech, Korea), 10 × IP-Taq Buffer (COSMO genetech, Korea), 2.5 U IP-Taq DNA polymerase (COSMO genetech, Korea), and the extracted DNA as the template. Deionized sterile distilled water (DW, Bioneer, Korea) was added to reach a final volume of 20 ㎕.

PCR conditions involved an initial 5 minutes pre-denaturation at 95°C, followed by 33 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. A final post-extension step was performed at 72°C for 7 minutes. The amplified products were visualized by electrophoresis on a 1% agarose gel containing SYBR™ Safe DNA Gel Stain (Invitrogen, USA) using 1 × TAE buffer (COSMO genetech, Korea). The PCR products were then detected under UV light.

Table 1. Primer set for analysis of *S. parauberis*

Target pathogen	Primer	Sequence (5'to 3')	Amplicon size (bp)	Reference
S. parauberis	pSP-F	TCCAGTCTTTCGACCTTCTT	220	Woo et al., 2006
	pSP-R	CAAAGAGATGTTCGGCTTG		

2.3.2. Serotype analysis of *S. parauberis*

Serotyping of *S. parauberis* was performed through PCR using the primer set proposed by Tu et al. (2015) and is shown in Table 2. In a 0.2 ㎖ microtube, a final PCR mixture of 20 ㎕ was prepared by adding 1 μM of each primer, 2.5 mM of each dNTP, 10 × IP-Taq Buffer, 2.5 U IP-Taq DNA polymerase, and the extracted DNA as the template. Distilled water was added to achieve a final volume of 20 ㎕.

PCR conditions included a 3 minute pre-denaturation at 95°C, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. A final post-extension step was performed at 72°C for 7 minutes. The amplified products were visualized by electrophoresis on a 1% agarose gel containing SYBR™ Safe DNA Gel Stain using 1 × TAE buffer. The PCR products were then detected under UV light.

Table 2. Primer sets for analysis of *S. parauberis* serotypes

Target serotype		Primer	Sequence (5'to 3')	Amplicon size (bp)	Reference
S. parauberis	subserotype Ia	For-Ia	ATTGTTAGTCATTCAGTTGT	213	Tu et al., 2015
		Rev-Ia	AATTATAGTCAACAGTCCAG		
	subserotype Ib/Ic	For-Ib/Ic	ATTTCTACCAGGTTACTTTG	303	
		Rev-Ib/Ic	ACATCTCGAAACTTCATATT		
	serotype II	For-II	GAACTACTTAGGTTTAGCAT	413	
		Rev-II	AACTTGTAAATAGGATTGCT		

2.4. Biochemical analysis

To analyze the biochemical characteristics based on serotypes, the API 20 STREP kit (BioMérieux, France) was employed in the experiment. 3 strains each of subserotype Ia, subserotype Ib/Ic, and serotype II were selected and cultured in 2% NaCl supplemented TSB at 28°C for 24 hours. After 24 hours of cultivation, the culture broth was used to conduct the API 20 STREP test.

An incubation box was prepared, and 5 ㎖ of sterile distilled water was dispensed into the tray. After placing the strip on the tray, 100 ㎕ of bacterial suspension was dispensed from sodium pyruvate (VP) to L-leucine-β-naphthylamide (LAP), and for L-arginine (ADH), the bacterial suspension was dispensed only into the tube. For D-ribose (RIB) to glycogen (GLYG), 0.5 ㎖ of bacterial suspension was mixed with the API GP medium ampoule and dispensed only into the tube. From ADH to GLYG, mineral oil was added to the cuvette, and the tubes were incubated at 37°C for 4 hours. After 4 hours, the incubation box was taken out, and reagents were added to VP, hippuric acid (HIP), pyroglutamic acid β-naphthylamide (PYRA), 6-bromo-2-naphthylαD-galactopyranoside (αGAL), naphthol ASBIglucuronic acid (βGUR), 2-naphthylβD-galactopyranoside (βGAL), 2-naphthyl phosphate (PAL), and LAP tests. 10 minutes later, the results were interpreted using a reading chart (Facklam et al., 1984; Human et al., 1985; MacGowan et al., 1989). The reactions for esculin (ESC), L-arginine (ADH), D-ribose (RIB), L-arabinose (ARA), D-mannitol (MAN), D-sorbitol (SOR), D-lactose (LAC), D-trehalose (TRE), inulin (INU), D-raffinose (RAF), starch (AMD), and GLYG, excluding enzyme reactions such as HIP, PYRA, αGAL, βGUR, βGAL, PAL, and LAP, were coded after incubation at 37°C for 24 hours. The code values were converted into a 7-digit numerical profile, and the results were interpreted using API identification software (*apiweb*™).

2.5. Antibiotic susceptibility test

Individual strains were cultivated in TSB at a temperature of 28°C for a duration of 24 hours. The culture was diluted to 10^5~10^6 for the antibiotic susceptibility test. The diluted solution was streaked on mueller hinton agar (MHA, Difco, USA) supplemented with 1% NaCl using a sterilized swab. 19 types of antibiotic disks (Liofilchem, Italy) were attached to the MHA, including β-lactam antibiotics (amoxicillin (AML), ampicillin (AMP), cephalexin (CL), ceftiofur (FUR)), aminoglycoside antibiotics (gentamycin (CN)), lincosamide antibiotics (clindamycin (CD), lincomycin (MY)), macrolide antibiotics (erythromycin (E)), tetracycline antibiotics (doxycycline (DXT), oxytetracycline (OT), tetracycline (TET)), sulfonamide antibiotics (sulfadiazine (SUZ), trimethoprim-sulfamethoxazole (SXT), trimethoprim (TM)), quinolone antibiotics (oxolinic acid (OA), nalidixic acid (NA), flumequine (UB), enrofloxacin (ENR)), and phenicol antibiotics (florfenicol (FFC)) (Table 3). The cultures were incubated at 28°C for 24 hours, and growth inhibition zones were examined.

Table 3. Disk used for antibiotic susceptibility test

Antibiotic classes	Name	Abbreviation	μg
β-lactam	Amoxicillin	AML	10
	Ampicillin	AMP	10
	Cephalexin	CL	30
	Ceftiofur	FUR	30
Aminoglycoside	Gentamycin	CN	10
Lincosamide	Clindamycin	CD	10
	Lincomycin	MY	15
Macrolide	Erythromycin	E	15
Tetracycline	Doxycycline	DXT	30
	Oxytetracycline	OT	30
	Tetracycline	TET	30
Sulfonamide	Sulfadiazine	SUZ	300
	Trimethoprim-Sulfamethoxazole	SXT	25
	Trimethoprim	TM	5
Quinolone	Oxolinic acidcin	OA	2
	Nalidixic acid	NA	30
	Flumequine	UB	30
	Enrofloxacin	ENR	5
Phenicol	Florfenicol	FFC	30

2.6. Tetracycline and erythromycin resistance gene detection

85 strains of *S. parauberis*, isolated in 2023, were used for the genetic analysis of antibiotic resistance genes. The investigation focused on 5 specific genes, namely *tet*(B), *tet*(M), *tet*(S), and *tet*(Y) from the tetracyclines, as well as *erm*(B) from the macrolides.

PCR was conducted using the primer sets delineated in Table 4. In a 0.2 ㎖ microtube, 1 μM of each primer, 2.5 mM of individual dNTPs, 10 × IP-Taq Buffer, 2.5 U IP-Taq DNA polymerase, and template DNA extracted from the strains were combined, and distilled water was added to achieve a final PCR mixture volume of 20 ㎕.

The annealing temperatures for *tet*(B), *tet*(M), and *tet*(S) were set at 55°C, while the annealing temperature for *tet*(Y) was set at 58°C. Additionally, the annealing temperature for *erm*(B) was set at 49°C. The PCR conditions involved a 3 minutes pre-denaturation at 95°C, followed by denaturation at 98°C for 10 seconds. Subsequently, annealing was conducted at the specified temperatures for 30 seconds according to the primer sets, followed by extension at 72°C for 30 seconds in one cycle. This cycle was repeated 30 times, and a final post-extension step was carried out at 72°C for 7 minutes. The amplified products were visualized by electrophoresis on a 1% agarose gel containing SYBR™ Safe DNA Gel Stain using 1 × TAE buffer. The PCR products were then detected under UV light.

Table 4. Primer sets for analysis of antibiotic resistance genes

Target gene	Primer pair	Sequence (5' to 3')	Amplicon size (bp)	Reference
tet(B)	TETF	GCGCTNTATGCGTTGATGCA	171	Jun et al., 2003
	TBR	TGAAAGCAAACGGCCTAA		
tet(M)	TMF	GAATCTGAACAATGGGAT	1,099	Jun et al., 2010
	TMR	CTAACAATTCTGTTCCAGC		
tet(S)	TSF	CATAGACAAGCCGTTGACC	667	
	TSR	ATGTTTTTGGAACGCCAGAG		
tet(Y)	*tet*(Y)-F	ATCACGCAAAGCTGGATGGT	278	Woo et al., 2021
	tet(Y)-R	GCTGACTGGCGCAATAATGG		
erm(B)	*erm*(B)-F	GAAAAGGTACTCAACCAAAT	639	Chung et al., 1999
	erm(B)-R	AGTAACGGTACTTAAATTGT		

2.7. Vaccine investigation containing antigens of *S. parauberis*

To investigate the serotype of the inactivated vaccine containing antigens of *S. parauberis* manufactured domestically, a vaccine approved as a veterinary medicine by the National Institute of Fisheries Science (NIFS) was examined. Details of vaccines containing antigens of *S. parauberis* targeting olive flounder are given in Table 5.

Table 5. Inactivated vaccines containing *S. parauberis* antigen in Korea

Company name	License year	Product name
㈜ 대성미생물연구소	2006.12.15	대성 연쇄 피쉬백
㈜ 코미팜	2009.03.30	프로백 더블에스
㈜ 고려비엔피	2009.12.08	힘백 어질방 S-3 플러스
㈜ 하나원	2010.01.26	포세이돈-3
㈜ 대성미생물연구소	2010.12.16	대성 연쇄 혼합 피쉬백
㈜ 하나원	2012.03.30	포세이돈-5
㈜ 코미팜	2012.07.25	프로백 에드와드연쇄상구균 혼합백신
㈜ 중앙백신연구소	2012.08.23	참신 연쇄 포커스
㈜ 코미팜	2015.01.12	프로백TM 펜타
㈜ 고려비엔피	2016.02.24	힘백 어질방 ES3 플러스 혼합 불활화 백신
㈜ 대성미생물연구소	2016.08.22	대성 VES-2 피쉬백
㈜ 고려비앤피	2018.04.11	월로마린 S.E 4
㈜ 씨티씨백	2022.03.03	씨티씨백 세균다자바 플러스

3. Results

3.1. Identification of *S. parauberis*

Strains identified as *S. parauberis*, isolated from diseased olive flounder in Jeju Island olive flounder farms from March 2019 to August 2023, are presented in Table 6. In 2019, 155 isolates were identified, and this was followed by 80 isolates in 2020, 30 isolates in 2021, 32 isolates in 2022, and 85 isolates in 2023. In total, 382 isolates were identified as *S. parauberis* (Table 6).

Table 6. Number of *S. parauberis* isolated from olive flounder in Jeju Island from 2019 to 2023

Year	2019	2020	2021	2022	2023	Total
No. of *S. parauberis*	155	80	30	32	85	382

3.2. Serotype analysis of *S. parauberis*

3.2.1. Serotype monitoring of *S. parauberis*

A serotype analysis was conducted on the 382 isolates identified as *S. parauberis*. Multiplex PCR was conducted using primer sets suggested by Tu et al (2015). The 382 isolates were classified into three serotypes (Fig. 2, Table 7).

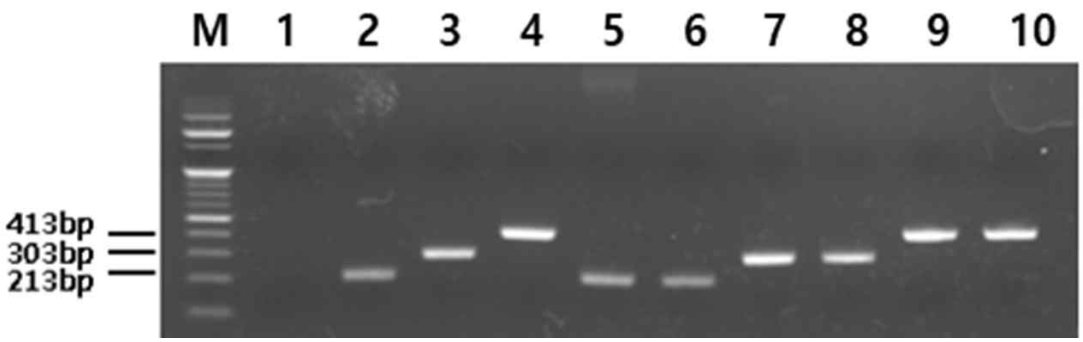

Fig. 2. Multiplex PCR products with the specific primer sets for *S. parauberis* serotypes. M, 100bp DNA ladder marker; lane 1, negative control; lane 2, positive control (subserotype Ia); lane 3, positive control (subserotype Ib/Ic); lane 4, positive control (serotype II); lane 5-6 subserotype Ia; lane 7-8 subserotype Ib/Ic; lane 9-10 serotype II.

Table 7. Serotype of *S. parauberis* isolates used in this study

Strains							
No.	Region	Year	Serotype	No.	Region	Year	Serotype
1	Pyoseon	2019	Ia	41	Gujwa	2019	Ia
2	Daejeong	2019	II	42	Namwon	2019	Ib/Ic
3	Daejeong	2019	Ia	43	Seongsan	2019	Ib/Ic
4	Pyoseon	2019	Ia	44	Seongsan	2019	Ib/Ic
5	Daejeong	2019	Ia	45	Daejeong	2019	Ia
6	Daejeong	2019	Ia	46	Daejeong	2019	Ia
7	Daejeong	2019	Ia	47	Seongsan	2019	Ib/Ic
8	Pyoseon	2019	Ia	48	Seongsan	2019	Ia
9	Gujwa	2019	Ib/Ic	49	Daejeong	2019	Ia
10	Pyoseon	2019	Ia	50	Daejeong	2019	Ia
11	Seongsan	2019	Ib/Ic	51	Daejeong	2019	Ia
12	Namwon	2019	Ib/Ic	52	Namwon	2019	Ib/Ic
13	Seongsan	2019	Ib/Ic	53	Seongsan	2019	Ib/Ic
14	Seongsan	2019	Ib/Ic	54	Daejeong	2019	Ib/Ic
15	Daejeong	2019	Ia	55	Gujwa	2019	Ia
16	Daejeong	2019	Ia	56	Namwon	2019	Ia
17	Pyoseon	2019	Ia	57	Seongsan	2019	Ia
18	Seongsan	2019	Ib/Ic	58	Daejeong	2019	Ib/Ic
19	Seongsan	2019	Ib/Ic	59	Seongsan	2019	Ia
20	Daejeong	2019	Ia	60	Seongsan	2019	Ia
21	Daejeong	2019	II	61	Seongsan	2019	Ia
22	Pyoseon	2019	Ia	62	Daejeong	2019	Ia
23	Namwon	2019	Ia	63	Namwon	2019	Ia
24	Gujwa	2019	Ib/Ic	64	Seongsan	2019	Ia
25	Daejeong	2019	Ia	65	Seongsan	2019	Ia
26	Gujwa	2019	Ib/Ic	66	Seongsan	2019	Ib/Ic
27	Daejeong	2019	Ia	67	Daejeong	2019	Ia
28	Daejeong	2019	Ia	68	Namwon	2019	Ia
29	Seongsan	2019	Ib/Ic	69	Seongsan	2019	Ib/Ic
30	Pyoseon	2019	Ia	70	Seongsan	2019	Ib/Ic
31	Pyoseon	2019	Ib/Ic	71	Seongsan	2019	Ia
32	Seongsan	2019	Ib/Ic	72	Seongsan	2019	Ib/Ic
33	Seongsan	2019	Ib/Ic	73	Seongsan	2019	Ia
34	Namwon	2019	Ia	74	Jocheon	2019	Ib/Ic
35	Namwon	2019	Ia	75	Seongsan	2019	Ib/Ic
36	Seongsan	2019	Ib/Ic	76	Seongsan	2019	Ib/Ic
37	Seongsan	2019	Ib/Ic	77	Seongsan	2019	Ia
38	Seongsan	2019	Ia	78	Daejeong	2019	Ia
39	Daejeong	2019	Ib/Ic	79	Seongsan	2019	Ib/Ic
40	Gujwa	2019	Ib/Ic	80	Jocheon	2019	Ia

Strains							
No.	Region	Year	Serotype	No.	Region	Year	Serotype
81	Seongsan	2019	Ib/Ic	121	Seongsan	2019	Ib/Ic
82	Seongsan	2019	Ia	122	Jocheon	2019	Ia
83	Daejeong	2019	Ia	123	Seongsan	2019	Ia
84	Seongsan	2019	Ib/Ic	124	Namwon	2019	Ia
85	Seongsan	2019	Ib/Ic	125	Seongsan	2019	Ia
86	Seongsan	2019	Ib/Ic	126	Seongsan	2019	Ib/Ic
87	Namwon	2019	Ia	127	Seongsan	2019	Ia
88	Seongsan	2019	Ib/Ic	128	Jocheon	2019	Ia
89	Daejeong	2019	II	129	Seongsan	2019	Ia
90	Daejeong	2019	Ib/Ic	130	Daejeong	2019	II
91	Seongsan	2019	Ib/Ic	131	Daejeong	2019	Ia
92	Namwon	2019	Ia	132	Seongsan	2019	Ia
93	Seongsan	2019	Ia	133	Namwon	2019	Ia
94	Seongsan	2019	Ia	134	Daejeong	2019	II
95	Seongsan	2019	Ib/Ic	135	Namwon	2019	Ia
96	Seongsan	2019	Ia	136	Pyoseon	2019	Ia
97	Seongsan	2019	Ib/Ic	137	Seongsan	2019	Ia
98	Seongsan	2019	Ib/Ic	138	Seongsan	2019	Ia
99	Seongsan	2019	Ib/Ic	139	Daejeong	2019	Ia
100	Seongsan	2019	Ia	140	Namwon	2019	Ia
101	Seongsan	2019	Ia	141	jocheon	2019	Ia
102	Namwon	2019	Ia	142	Seongsan	2019	Ia
103	Namwon	2019	Ia	143	Daejeong	2019	Ia
104	Namwon	2019	Ia	144	Seongsan	2019	Ia
105	Seongsan	2019	Ia	145	Namwon	2019	Ia
106	Seongsan	2019	Ia	146	Seongsan	2019	Ia
107	Seongsan	2019	Ia	147	Seongsan	2019	II
108	Seongsan	2019	Ia	148	Daejeong	2019	II
109	Seongsan	2019	Ib/Ic	149	Seongsan	2019	Ib/Ic
110	Namwon	2019	Ia	150	Namwon	2019	Ia
111	Namwon	2019	Ia	151	Seongsan	2019	Ia
112	Daejeong	2019	Ia	152	Seongsan	2019	II
113	Seongsan	2019	Ia	153	Seongsan	2019	II
114	Seongsan	2019	Ib/Ic	154	Seongsan	2019	Ia
115	Namwon	2019	II	155	Gujwa	2019	Ia
116	Seongsan	2019	Ia	156	Daejeong	2020	II
117	Daejeong	2019	II	157	Namwon	2020	Ia
118	Namwon	2019	Ia	158	Daejeong	2020	Ib/Ic
119	Seongsan	2019	Ia	159	Seongsan	2020	Ia
120	Seongsan	2019	Ib/Ic	160	Namwon	2020	Ia

Strains							
No.	Region	Year	Serotype	No.	Region	Year	Serotype
161	Seongsan	2020	Ia	201	Seongsan	2020	Ia
162	Namwon	2020	Ia	202	Daejeong	2020	Ia
163	Seongsan	2020	Ia	203	Seongsan	2020	Ia
164	Seongsan	2020	Ia	204	Daejeong	2020	Ia
165	Namwon	2020	Ia	205	Seongsan	2020	Ia
166	Daejeong	2020	Ia	206	Namwon	2020	Ia
167	Jocheon	2020	Ia	207	Daejeong	2020	Ia
168	Gujwa	2020	Ia	208	Gujwa	2020	Ia
169	Seongsan	2020	Ia	209	Gujwa	2020	Ia
170	Namwon	2020	Ia	210	Namwon	2020	Ia
171	Daejeong	2020	Ib/Ic	211	Seongsan	2020	Ia
172	Daejeong	2020	Ia	212	Daejeong	2020	Ia
173	Seongsan	2020	Ia	213	Seongsan	2020	Ia
174	Namwon	2020	II	214	Gujwa	2020	Ia
175	Seongsan	2020	Ia	215	Gujwa	2020	Ia
176	Seongsan	2020	Ia	216	Seongsan	2020	Ia
177	Namwon	2020	Ia	217	Seongsan	2020	Ia
178	Seongsan	2020	Ia	218	Daejeong	2020	Ib/Ic
179	Daejeong	2020	Ia	219	Gujwa	2020	Ia
180	Seongsan	2020	Ia	220	Gujwa	2020	Ia
181	Daejeong	2020	Ia	221	Gujwa	2020	Ia
182	Namwon	2020	Ia	222	Gujwa	2020	Ia
183	Daejeong	2020	Ia	223	Seongsan	2020	Ia
184	Daejeong	2020	Ia	224	Gujwa	2020	Ia
185	Seongsan	2020	Ia	225	Gujwa	2020	Ia
186	Namwon	2020	Ia	226	Daejeong	2020	Ia
187	Seongsan	2020	Ia	227	Seongsan	2020	Ia
188	Daejeong	2020	Ia	228	Daejeong	2020	Ia
189	Daejeong	2020	Ia	229	Daejeong	2020	Ia
190	Seongsan	2020	Ia	230	Seongsan	2020	Ia
191	Daejeong	2020	Ia	231	Gujwa	2020	Ia
192	Jocheon	2020	Ia	232	Seongsan	2020	Ia
193	Seongsan	2020	Ia	233	Namwon	2020	Ia
194	Seongsan	2020	Ia	234	Seongsan	2020	Ia
195	Namwon	2020	Ia	235	Seongsan	2020	Ia
196	Daejeong	2020	Ia	236	Seongsan	2021	Ia
197	Namwon	2020	Ia	237	Jocheon	2021	Ia
198	Daejeong	2020	Ia	238	Daejeong	2021	Ia
199	Daejeong	2020	Ia	239	Daejeong	2021	Ia
200	Namwon	2020	Ia	240	Daejeong	2021	Ia

Strains							
No.	Region	Year	Serotype	No.	Region	Year	Serotype
241	Seongsan	2021	Ia	281	Namwon	2022	Ia
242	Seongsan	2021	Ia	282	Gujwa	2022	Ia
243	Daejeong	2021	Ia	283	Gujwa	2022	Ia
244	Seongsan	2021	II	284	Daejeong	2022	II
245	Pyoseon	2021	Ia	285	Pyoseon	2022	Ia
246	Seongsan	2021	Ia	286	Pyoseon	2022	Ia
247	Daejeong	2021	Ia	287	Seongsan	2022	II
248	Jocheon	2021	Ia	288	Seongsan	2022	Ia
249	Seongsan	2021	Ia	289	Pyoseon	2022	Ia
250	Seongsan	2021	Ib/Ic	290	Gujwa	2022	Ia
251	Gujwa	2021	Ib/Ic	291	Gujwa	2022	II
252	Seongsan	2021	Ia	292	Seongsan	2022	II
253	Seongsan	2021	Ia	293	Jocheon	2022	Ia
254	Gujwa	2021	Ia	294	Seongsan	2022	II
255	Jocheon	2021	Ia	295	Daejeong	2022	II
256	Seongsan	2021	II	296	Seongsan	2022	II
257	Daejeong	2021	Ia	297	Daejeong	2022	Ia
258	Seongsan	2021	Ib/Ic	298	Seongsan	2023	II
259	Gujwa	2021	Ib/Ic	299	Namwon	2023	II
260	Seongsan	2021	II	300	Namwon	2023	II
261	Daejeong	2021	II	301	Daejeong	2023	Ia
262	Seongsan	2021	II	302	Daejeong	2023	Ia
263	Gujwa	2021	Ia	303	Daejeong	2023	Ia
264	Seongsan	2021	Ia	304	Daejeong	2023	Ia
265	Seongsan	2021	II	305	Daejeong	2023	Ia
266	Daejeong	2022	Ia	306	Daejeong	2023	Ia
267	Daejeong	2022	Ia	307	Daejeong	2023	Ia
268	Daejeong	2022	Ia	308	Daejeong	2023	Ia
269	Seongsan	2022	II	309	Daejeong	2023	Ia
270	Seongsan	2022	Ia	310	Daejeong	2023	Ia
271	Seongsan	2022	Ia	311	Daejeong	2023	Ia
272	Seongsan	2022	Ia	312	Daejeong	2023	Ia
273	Daejeong	2022	Ia	313	Hangyeong	2023	Ia
274	Daejeong	2022	Ia	314	Daejeong	2023	Ia
275	Seongsan	2022	II	315	Hangyeong	2023	Ia
276	Seongsan	2022	Ia	316	Hangyeong	2023	Ia
277	Seongsan	2022	Ia	317	Seongsan	2023	Ia
278	Gujwa	2022	II	318	Seongsan	2023	Ia
279	Seongsan	2022	Ia	319	Seongsan	2023	Ia
280	Seongsan	2022	Ia	320	Seongsan	2023	Ia

Strains							
No.	Region	Year	serotype	No.	Region	Year	serotype
321	Daejeong	2023	Ia	352	Jocheon	2023	II
322	Hangyeong	2023	Ia	353	Jocheon	2023	II
323	Hangyeong	2023	Ia	354	Jocheon	2023	II
324	Hangyeong	2023	Ia	355	Jocheon	2023	II
325	Hangyeong	2023	Ia	356	Jocheon	2023	II
326	Hangyeong	2023	Ia	357	Namwon	2023	Ia
327	Hangyeong	2023	Ia	358	Namwon	2023	Ia
328	Namwon	2023	Ia	359	Namwon	2023	Ia
329	Namwon	2023	Ia	360	Namwon	2023	Ia
330	Seongsan	2023	Ia	361	Daejeong	2023	Ia
331	Daejeong	2023	II	362	Daejeong	2023	Ia
332	Seongsan	2023	Ia	363	Namwon	2023	Ia
333	Seongsan	2023	Ia	364	Namwon	2023	Ia
334	Seongsan	2023	Ia	365	Gujwa	2023	Ia
335	Seongsan	2023	Ia	366	Daejeong	2023	Ia
336	Seongsan	2023	Ia	367	Daejeong	2023	Ia
337	Seongsan	2023	Ia	368	Daejeong	2023	Ia
338	Daejeong	2023	Ia	369	Daejeong	2023	Ia
339	Daejeong	2023	II	370	Daejeong	2023	Ia
340	Daejeong	2023	II	371	Daejeong	2023	Ia
341	Daejeong	2023	Ib/Ic	372	Daejeong	2023	Ia
342	Daejeong	2023	Ia	373	Daejeong	2023	Ia
343	Daejeong	2023	Ia	374	Daejeong	2023	Ib/Ic
344	Gujwa	2023	Ia	375	Daejeong	2023	Ib/Ic
345	Gujwa	2023	Ia	376	Seongsan	2023	II
346	Hangyeong	2023	Ia	377	Seongsan	2023	II
347	Hangyeong	2023	Ia	378	Hangyeong	2023	Ib/Ic
348	Hangyeong	2023	Ia	379	Hangyeong	2023	Ia
349	Hangyeong	2023	Ia	380	Daejeong	2023	Ia
350	Hangyeong	2023	Ia	381	Hangyeong	2023	Ia
351	Seongsan	2023	Ia	382	Daejeong	2023	Ia

3.2.2. *S. parauberis* serotype monitoring by year and region

The trend of detecting three serotypes of *S. parauberis* collected between March 2019 and August 2023 is presented in Table 8. Among 382 isolates, subserotype Ia accounted for 279 isolates (73%), subserotype Ib/Ic for 61 isolates (16%), and serotype II for 42 isolates (11%) (Fig. 3A). Subserotype Ia consistently showed high detection rates each year. The detection rate was higher than that of subserotype Ib/Ic and serotype II every year, at 61% in 2019, 94% in 2020, 67% in 2021, 69% in 2022, and 80% in 2023. (Fig. 3B, Table 8). Subserotype Ib/Ic decreased significantly from 32% in 2019 to 4% in 2020, and further to 0% in 2022, with a slight increase to 5% in 2023 (Fig. 3B, Table 8). Serotype II showed an increasing trend from 3% in 2020 to 20% in 2021, 31% in 2022, and 15% in 2023 (Fig. 3B, Table 8).

Although the same isolates were not consistently collected from the same fish farms each year, the results were organized the results to analyze the pattern of serotype detection (Fig. 4, Table 9, Table 10, Table 11, Table 12, Table 13, Table 14). From 2019 to 2022, no olive flounder was collected from fish farms in Hangyeong, and in 2020, there were no olive flounder was collected from both Hangyeong and Pyoseon fish farms. In 2021, no olive flounder was collected from Hangyeong and Namwon fish farms, and in 2023, no olive flounder was collected from the Pyoseon fish farms (Fig. 4).

In 2019, a total of 155 isolates of *S. parauberis* were collected from Seongsan (76 isolates), Daejeong (34 isolates), Namwon (25 isolates), Pyoseon (8 isolates), Gujwa (7 isolates), and Jocheon (5 isolates) areas of olive flounder farms. There were 94 isolates of subserotype Ia, 50 isolates of subserotype Ib/Ic, and 11 isolates of serotype II detected, with subserotype Ia being the most frequently detected serotype in 2019 (Fig. 3, Table 9, Table 10). In 2020, a total of 80 isolates of *S. parauberis* were collected from Seongsan (28 isolates), Daejeong (23 isolates), Namwon (15 isolates), Gujwa

(11 isolates), and Jocheon (3 isolates) areas of olive flounder farms. There were 75 isolates of subserotype Ia, 3 isolates of subserotype Ib/Ic, and 2 isolates of serotype II detected, with subserotype Ia being the most frequently detected serotype in 2020 (Fig. 3, Table 9, Table 11). In 2021, a total of 30 isolates of *S. parauberis* were collected. They were detected in Seongsan (15 isolates), Daejeong (7 isolates), Gujwa (4 isolates), Jocheon (3 isolates), and Pyoseon (1 isolate) areas of olive flounder farms. There were 20 isolates of subserotype Ia, 4 isolates of subserotype Ib/Ic, and 6 isolates of serotype II detected, with subserotype Ia being the most frequently detected serotype in 2021 (Fig. 3, Table 9, Table 12). In 2022, a total of 32 isolates of *S. parauberis* were collected. They were detected in Seongsan (14 isolates), Daejeong (8 isolates), Gujwa (5 isolates), Pyoseon (3 isolates), Jocheon, and Namwon (1 isolate each) areas of olive flounder farms. There were 22 isolates of subserotype Ia, and no subserotype Ib/Ic were detected. Additionally, 10 isolates of serotype II were detected, with subserotype Ia being the most detected serotype in 2022 (Fig. 3, Table 9, Table 13). In 2023, a total of 85 isolates of *S. parauberis* were collected. They were detected in Daejeong (35 isolates), Hangyeong (17 isolates), Seongsan (15 isolates), Namwon (10 isolates), Jocheon (5 isolates), and Gujwa (3 isolates) areas of olive flounder farms. Among them, 67 isolates were identified as subserotype Ia, 4 isolates as subserotype Ib/Ic, and 14 isolates as serotype II, with subserotype Ia being the most frequently detected serotype in 2023 (Fig. 3, Table 9, Table 14).

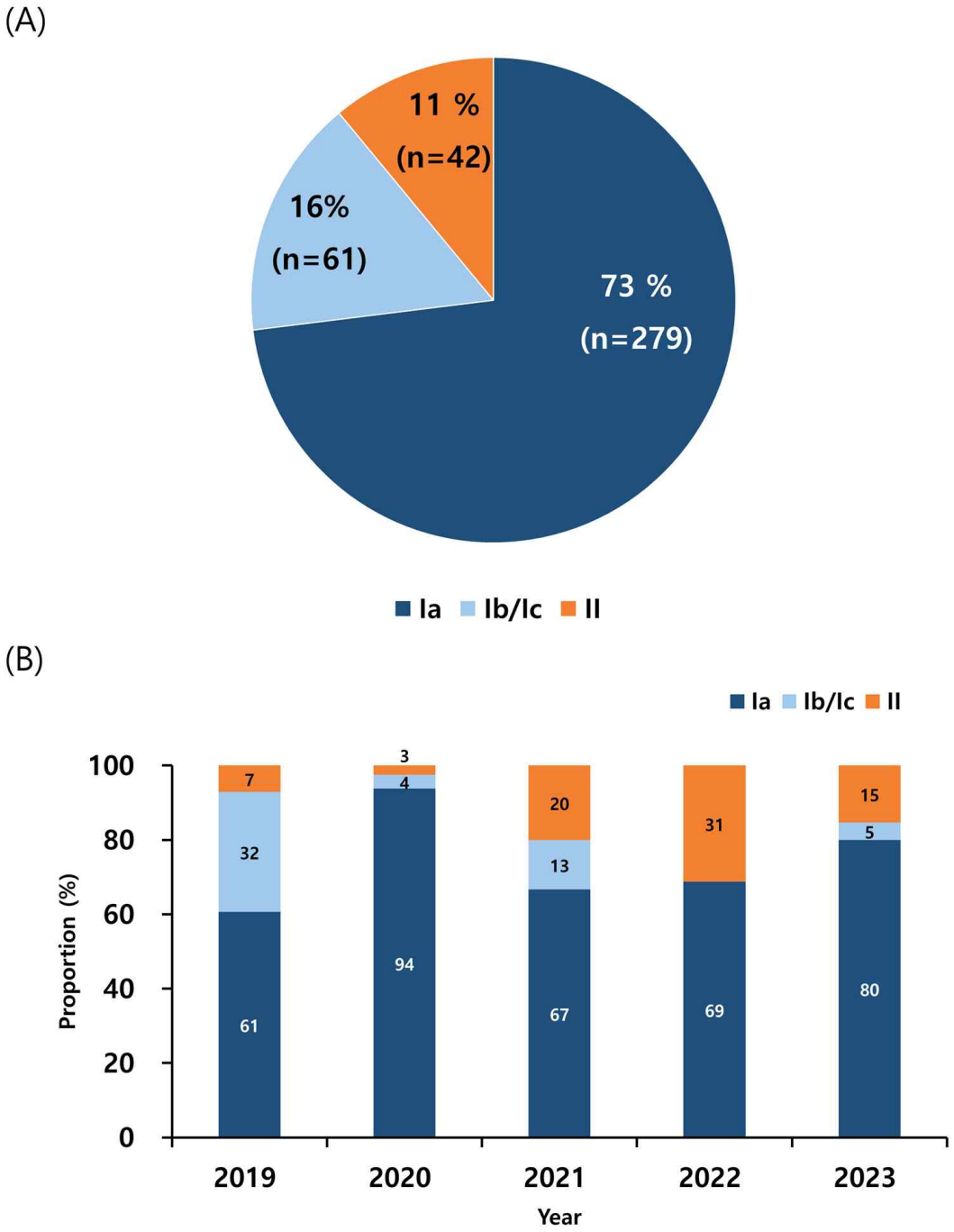

Fig. 3. Number of serotype isolates of *S. parauberis* detected from 2019 to 2023. (A) total number of *S. parauberis* serotype isolates detected from 2019 to 2023; (B) number of serotype strains of *S. parauberis* by year.

Table 8. Number of *S. parauberis* serotype isolates detected from farmed olive flounder in Jeju Island from 2019 to 2023

Serotype \ Year	2019	2020	2021	2022	2023	Total
Ia	94	75	20	22	68	279
Ib/Ic	50	3	4	0	4	61
II	11	2	6	10	13	42
Total	155	80	30	32	85	382

Table 9. Number of *S. parauberis* strains isolated by region and year

Year \ Region	H.G	J.C	G.J	D.J	N.W	P.S	S.S	Total
2019	-	5	7	34	25	8	76	155
2020	-	3	11	23	15	-	28	80
2021	-	3	4	7	-	1	15	30
2022	-	1	5	8	1	3	14	32
2023	17	5	3	35	10	-	15	85
Total	17	17	30	107	51	12	148	382

H.G, Hangyeong; J.C, Jocheon; G.J, Gujwa; D.J, Daejeong; N.W, Namwon; P.S, Pyoseon; S.S, Seongsan; -, not collected in the specified region.

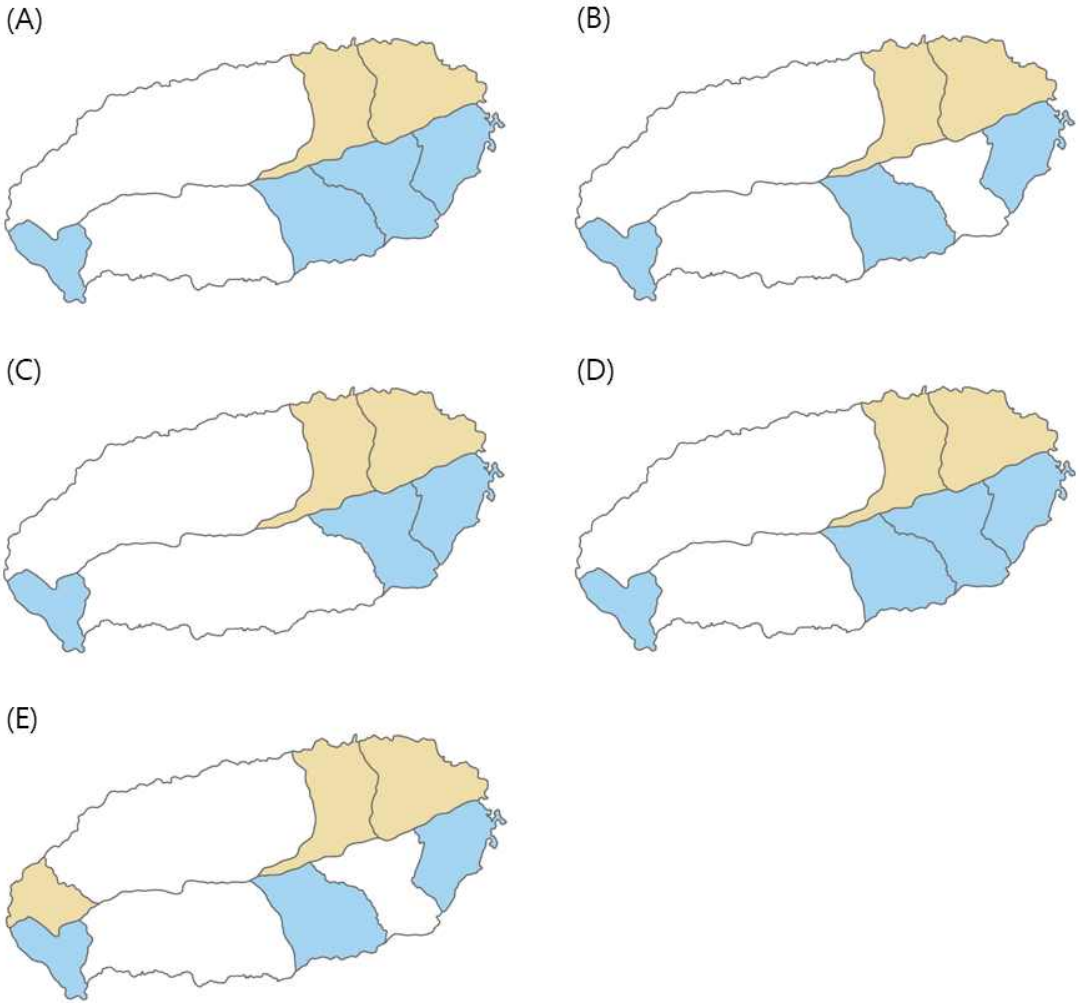

Fig. 4. Location of the sampling sites from 2019 to 2023. (A) location of the sampling sites in 2019; (B) location of the sampling sites in 2020; (C) location of the sampling sites in 2021; (D) location of the sampling sites in 2022; (E) location of the sampling sites in 2023. yellow area; jeju-si olive flounder farms; bule area, seogwipo-si olive flounder farms.

Table 10. Number of *S. parauberis* serotype isolates detected in olive flounder in Jeju Island in 2019

Year	Month	No. of *S. parauberis*	Serotype	Region						
				H.G	J.C	G.J	D.J	N.W	P.S	S.S
2019	Mar.	3	Ia	-			1		1	
			Ib/Ic	-						
			II	-			1			
	Apr.	23	Ia	-			7	1	5	
			Ib/Ic	-		3		1		5
			II	-			1			
	May.	25	Ia	-			7	3	1	2
			Ib/Ic	-		2	1	1		8
			II	-						
	Jun.	20	Ia	-			3	4		6
			Ib/Ic	-		1	1			5
			II	-						
	Jul.	31	Ia	-	1		2	3		8
			Ib/Ic	-	1		1			14
			II	-			1			
	Aug.	14	Ia	-			1	4		6
			Ib/Ic	-						2
			II	-				1		
	Sep.	13	Ia	-	2			2		5
			Ib/Ic	-						3
			II	-			1			
	Oct.	9	Ia	-			1	2	1	3
			Ib/Ic	-						
			II	-			2			
	Nov.	4	Ia	-	1		1	1		1
			Ib/Ic	-						
			II	-						
	Dec.	13	Ia	-		1	1	2		4
			Ib/Ic	-						1
			II	-			1			3
Total		155	Ia (n=94)	-	4	1	24	22	8	35
			Ib/Ic (n=50)	-	1	6	3	2	0	38
			II (n=11)	-	0	0	7	1	0	3

H.G, Hangyeong; J.C, Jocheon; G.J, Gujwa; D.J, Daejeong; N.W, Namwon; P.S, Pyoseon; S.S, Seongsan; -, not collected in the specified region.

Table 11. Number of *S. parauberis* serotype isolates detected in olive flounder in Jeju Island in 2020

Year	Month	No. of *S. parauberis*	Serotype	Region						
				H.G	J.C	G.J	D.J	N.W	P.S	S.S
2020	Feb.	5	Ia	-				2	-	1
			Ib/Ic	-			1		-	
			II	-			1		-	
	Mar.	11	Ia	-	1	1	1	3	-	4
			Ib/Ic	-			1		-	
			II	-					-	
	May.	9	Ia	-			2	1	-	5
			Ib/Ic	-					-	
			II	-				1	-	
	Jun.	7	Ia	-			3	2	-	2
			Ib/Ic	-					-	
			II	-					-	
	Jul.	3	Ia	-			2		-	1
			Ib/Ic	-					-	
			II	-					-	
	Aug.	9	Ia	-	1		4	2	-	2
			Ib/Ic	-					-	
			II	-					-	
	Sep.	7	Ia	-			2	2	-	3
			Ib/Ic	-					-	
			II	-					-	
	Oct.	15	Ia	-	1	7	2	1	-	4
			Ib/Ic	-			1		-	
			II	-					-	
	Nov.	5	Ia	-		2	1		-	2
			Ib/Ic	-					-	
			II	-					-	
	Dec.	8	Ia	-		1	2	1	-	4
			Ib/Ic	-					-	
			II	-					-	
Total		80	Ia (n=75)	-	3	11	19	14	-	28
			Ib/Ic (n=3)	-	0	0	3	0	-	0
			II (n=2)	-	0	0	1	1	-	0

H.G, Hangyeong; J.C, Jocheon; G.J, Gujwa; D.J, Daejeong; N.W, Namwon; P.S, Pyoseon; S.S, Seongsan; -, not collected in the specified region.

Table 12. Number of *S. parauberis* serotype isolates detected in olive flounder in Jeju Island in 2021

Year	Month	No. of *S. parauberis*	Serotype	Region						
				H.G	J.C	G.J	D.J	N.W	P.S	S.S
2021	Jan.	1	Ia	-				-		1
			Ib/Ic	-				-		
			II	-				-		
	Feb.	2	Ia	-	1		1	-		
			Ib/Ic	-				-		
			II	-				-		
	Apr.	3	Ia	-			2	-		1
			Ib/Ic	-				-		
			II	-				-		
	Jun.	2	Ia	-			1	-		1
			Ib/Ic	-				-		
			II	-				-		
	Jul.	12	Ia	-	2	1	1	-	1	4
			Ib/Ic	-		1		-		1
			II	-				-		1
	Aug.	4	Ia	-			1	-		
			Ib/Ic	-		1		-		1
			II	-				-		1
	Sep.	3	Ia	-				-		
			Ib/Ic	-				-		
			II	-			1	-		2
	Oct.	3	Ia	-		1		-		1
			Ib/Ic	-				-		
			II	-				-		1
Total		30	Ia (n=20)	-	3	2	6	-	1	8
			Ib/Ic (n=4)	-	0	2	0	-	0	2
			II (n=6)	-	0	0	1	-	0	5

H.G, Hangyeong; J.C, Jocheon; G.J, Gujwa; D.J, Daejeong; N.W, Namwon; P.S, Pyoseon; S.S, Seongsan; -, not collected in the specified region.

Table 13. Number of *S. parauberis* serotype isolates detected in olive flounder in Jeju Island in 2022

Year	Month	No. of *S. parauberis*	Serotype	Region						
				H.G	J.C	G.J	D.J	N.W	P.S	S.S
2022	Jan.	1	Ia	-			1			
			Ib/Ic	-						
			II	-						
	Feb.	1	Ia	-			1			
			Ib/Ic	-						
			II	-						
	Mar.	4	Ia	-			1			2
			Ib/Ic	-						
			II	-						1
	Apr.	6	Ia	-			2			3
			Ib/Ic	-						
			II	-						1
	May.	6	Ia	-		2		1		2
			Ib/Ic	-						
			II	-		1				
	Sep.	9	Ia	-		1			3	1
			Ib/Ic	-						
			II	-		1	1			2
	Oct.	4	Ia	-	1					
			Ib/Ic	-						
			II	-			1			2
	Nov.	8	Ia	-			1			
			Ib/Ic	-						
			II	-						
Total		32	Ia (n=22)	-	1	3	6	1	3	8
			Ib/Ic (n=0)	-	0	0	0	0	0	0
			II (n=10)	-	0	2	2	0	0	6

H.G, Hangyeong; J.C, Jocheon; G.J, Gujwa; D.J, Daejeong; N.W, Namwon; P.S, Pyoseon; S.S, Seongsan; -, not collected in the specified region.

Table 14. Number of *S. parauberis* serotype isolates detected in olive flounder in Jeju Island in 2023

Year	Month	No. of *S. parauberis*	Serotype	Region						
				H.G	J.C	G.J	D.J	N.W	P.S	S.S
2023	Jan.	19	Ia	3			13		-	
			Ib/Ic						-	
			II					2	-	1
	Feb.	14	Ia	6			1	2	-	5
			Ib/Ic						-	
			II						-	
	Mar.	23	Ia	5		2	3		-	7
			Ib/Ic				1		-	
			II		2		3		-	
	Apr.	7	Ia					3	-	
			Ib/Ic						-	
			II		3			1	-	
	May.	2	Ia				2		-	
			Ib/Ic						-	
			II						-	
	Jun.	13	Ia			1	8	2	-	
			Ib/Ic				2		-	
			II						-	
	Jul.	4	Ia	1					-	
			Ib/Ic	1					-	
			II						-	2
	Aug.	3	Ia	1			2		-	
			Ib/Ic						-	
			II						-	
Total		85	Ia (n=67)	16	0	3	29	7	-	12
			Ib/Ic (n=4)	1	0	0	3	0	-	0
			II (n=14)	0	5	0	3	3	-	3

H.G, Hangyeong; J.C, Jocheon; G.J, Gujwa; D.J, Daejeong; N.W, Namwon; P.S, Pyoseon; S.S, Seongsan; -, not collected in the specified region.

3.3. Biochemical analysis

Presents the results of the biochemical analysis for the 9 strains used in the experiment (Table 15). All 9 strains used for isolation showed enzymatic activity in the VP test for acetoin production, HIP test for hippuric acid hydrolysis, and LAP test for leucine aminopeptidase. No enzymatic activity was observed in the PYRA, αGAL, βGUR, βGAL, PAL, LAP, ADH, RIB, ARA, MAN, SOR, LAC, TRE, RAF, AMD, and GLYG tests. Only isolate 1 of subserotype Ia exhibited acidification in the INU test. Except for the results of the INU test, the biochemical profiles were similar among the 9 isolates, and the reaction of isolate 1 of subserotype Ia in the INU test was not considered indicative of distinguishing serotypes. No biochemical reactions capable of distinguishing serotype was observed.

Table 15. Biochemical activity of *S. parauberis* in API 20 STREP

Strain / Test	*S. parauberis*								
	Ia			Ib/Ic			II		
	1	2	3	1	2	3	1	2	3
VP	+	+	+	+	+	+	+	+	+
HIP	+	+	+	+	+	+	+	+	+
ESC	−	−	−	−	−	−	−	−	−
PYRA	−	−	−	−	−	−	−	−	−
αGAL	−	−	−	−	−	−	−	−	−
βGUR	−	−	−	−	−	−	−	−	−
βGAL	−	−	−	−	−	−	−	−	−
PAL	−	−	−	−	−	−	−	−	−
LAP	+	+	+	+	+	+	+	+	+
ADH	−	−	−	−	−	−	−	−	−
RIB	−	−	−	−	−	−	−	−	−
ARA	−	−	−	−	−	−	−	−	−
MAN	−	−	−	−	−	−	−	−	−
SOR	−	−	−	−	−	−	−	−	−
LAC	−	−	−	−	−	−	−	−	−
TRE	−	−	−	−	−	−	−	−	−
INU	+	−	−	−	−	−	−	−	−
RAF	−	−	−	−	−	−	−	−	−
AMD	−	−	−	−	−	−	−	−	−
GLYG	−	−	−	−	−	−	−	−	−

VP, acetoin production; HIP, hippurate hydrolysis; ESC, β-glucosidase hydrolysis; PYRA, pyrrolidonyl arylamidase; αGAL, α-galactosidase; βGUR, β-giucuronidase; βGAL, β-galactosidase; PAL, alkaline phosphatase; LAP, leucine arylamidase; ADH, arginine dihydrolase; RIB, acidification from ribose; ARA, acidification from arabinose; MAN, acidification from mannitol; SOR, acidification from sorbitol; LAC, acidification from lactose; TRE, acidification from trehalose; INU, acidification from inulin; RAF, acidification from raffinose; AMD, acidification from amidon; AMD, acidification from starch; GLYG, acidification from glycogen; −, negative; +, positive.

3.4. Antibiotic susceptibility

In 2023, antibiotic susceptibility testing was conducted on isolated of *S. parauberis* using 19 different antibiotic disks, and the inhibition zones were measured using the disc diffusion method. Among 67 isolates of suberotype Ia, 65 isolates excluding 2 isolates, 4 isolates of subserotype Ib/Ic, and 13 isolates of serotype II were used in the disk diffusion test method. The antibiotic susceptibility results for a total of 83 isolates (Fig. 5). No significant differences were observed among the three serotypes.

Among β-lactam antibiotics, 13% of isolates exhibited zones of 6mm or less for amoxicillin (AML), 14% for ampicillin (AMP), 7% for cephalexin (CL), and 4% for ceftiofur (FUR) (Fig. 5A). For aminoglycosides, 40% of isolates showed zones of 6mm or less for gentamicin (CN) (Fig. 5B). In the lincosamide antibiotics, clindamycin (CD) and lincomycin (MY) displayed zones of 6mm or less in 11% and 14% of isolates, respectively (Fig. 5C). The macrolide antibiotic erythromycin (E) showed zones of 6mm or less in 22% of isolates (Fig. 5D). Tetracycline antibiotics doxycycline (DXT), oxytetracycline (OT), and tetracycline (TET) exhibited zones of 6mm or less in 11%, 29%, and 14% of isolates, respectively (Fig. 5E). Sulfonamide antibiotics sulfadiazine (SUZ), trimethoprim-sulfamethoxazole (SXT), and trimethoprim (TM) showed zones of 6mm or less in 82%, 23%, and 8% of isolates, respectively (Fig. 5F). Among quinolone antibiotics, oxolinic acid (OA), nalidixic acid (NA), flumequine (UB), and enrofloxacin (ENR) exhibited zones of 6mm or less in 89%, 83%, 87%, and 6% of isolates, respectively (Fig. 5G). The phenicol antibiotic florfenicol (FFC) showed zones of 6mm or less in 11% of isolates (Fig. 5H).

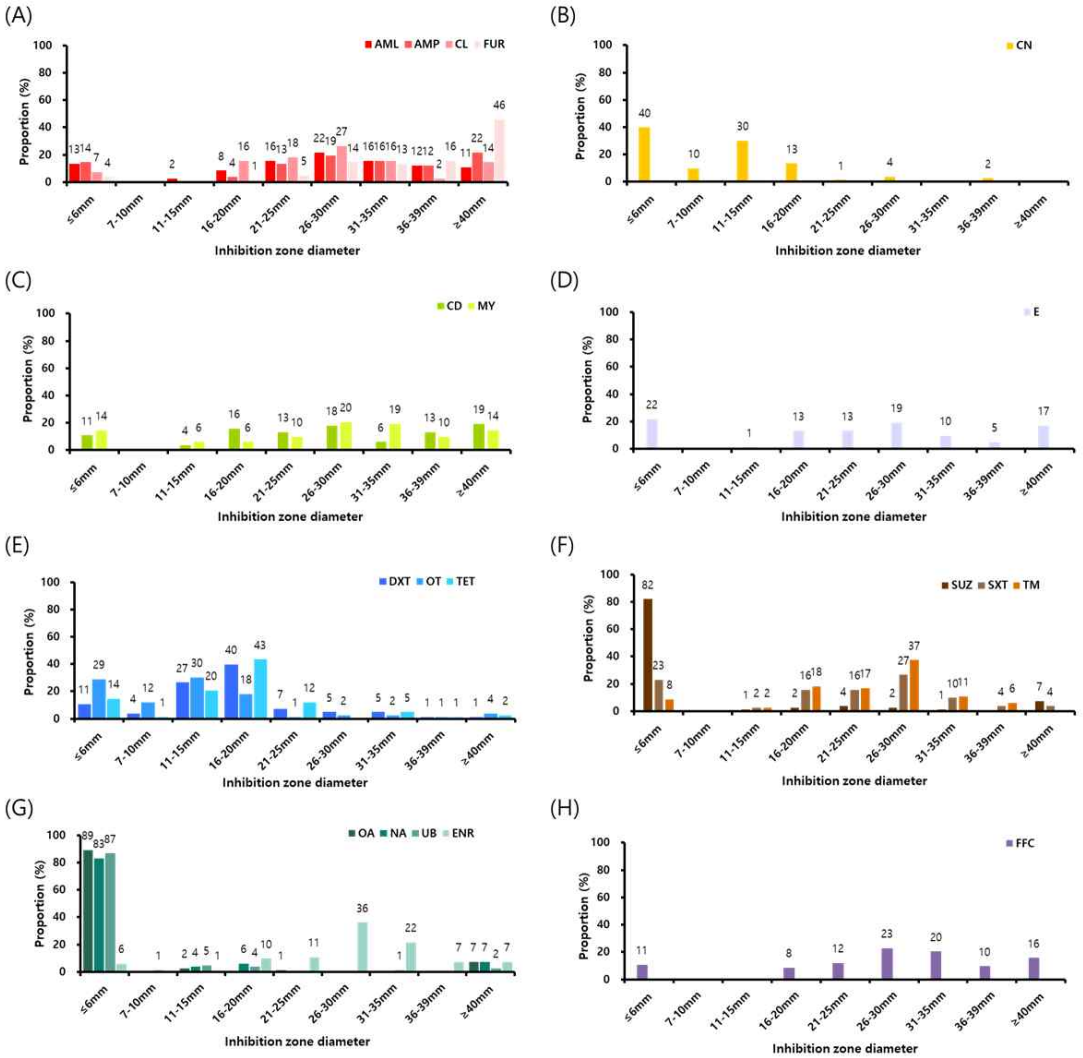

Fig. 5. Disk diffusion results for antibiotics of *S. parauberis* serotype. (A) β-lactam antibiotics, AML, amoxicillin; AMP, ampicillin; CL, cephalexin; FUR, ceftiofur; (B) aminoglycoside antibiotics, CN, gentamycin; (C) lincosamide antibiotics, CD, clindamycin; MY, lincomycin; (D) macrolide antibiotics, E, erythromycin; (E) tetracycline antibiotics, DXT, doxycycline; OT, oxytetracycline; TET, tetracycline; (F) sulfonamide antibiotics, SUZ, sulfadiazine; SXT, trimethoprim-sulfamethoxazole; TM, trimethoprim; (G) quinolone antibiotics, OA, oxolinic acid; NA, nalidixic acid; UB, flumequine; ENR, enrofloxacin; (H) phenicol antibiotics, FFC, florfenicol.

3.5. Tetracycline and erythromycin resistance gene analysis

In the antibiotic resistance gene analysis conducted in 2023 on the 85 isolates of *S. parauberis* that were identified, it was observed that the detection patterns of antibiotic resistance genes varied according to the serotype (Fig. 6, Table 16). Among the 67 isolates corresponding to subserotype Ia, *tet*(S) was detected in 67 isolates, *erm*(B) was detected in 13 isolates, and 13 isolates were found to have both *erm*(B) and *tet*(S). None of the 4 isolates corresponding to subserotype Ib/Ic showed the presence of antibiotic resistance genes. Among the 14 isolates corresponding to serotype II, *tet*(M) was detected in 13 isolates. The genes *tet*(B) and *tet*(Y) were not detected in isolates of subserotype Ia, subserotype Ib/Ic, and serotype II.

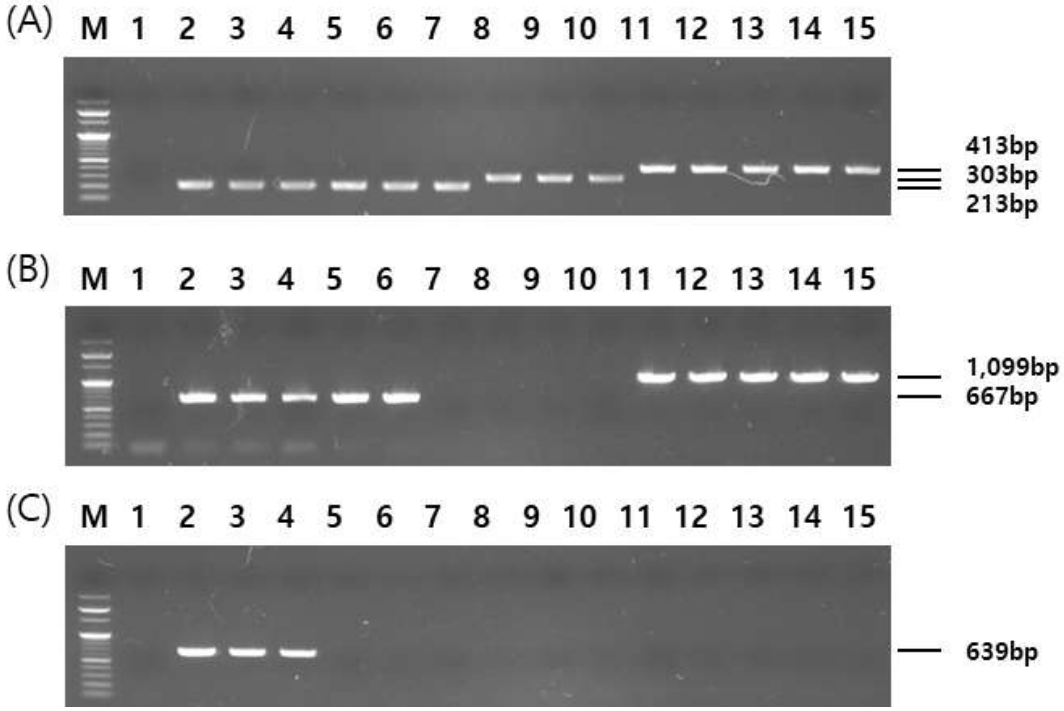

Fig. 6. PCR products with the specific primer sets. (A) PCR products with the specific primer sets for serotypes; (B) PCR products with the specific primer sets for *tet*(M), *tet*(S); (C) PCR products with the specific primer set for *erm*(B); M, 100bp DNA ladder marker; lane 1, negative control; lane 2-7, subserotype Ia; lane 8-10, subserotype Ib/Ic; lane 11-15, serotype II.

Table 16. Correlation of serotypes and antibiotic resistance genes in *S. parauberis*

serotype	Antibiotic resistance genes				
	tet(B)	*tet*(M)	*tet*(S)	*tet*(Y)	*erm*(B)
Ia (n=67)	0[1]	0	67	0	13
Ib/Ic (n=4)	0	0	0	0	0
II (n=14)	0	13	0	0	0

[1]No antibiotic resistance gene detected.

3.6. Serotypes of vaccines containing *S. parauberis* antigens

The serotypes of domestically produced inactivated vaccines containing *S. parauberis* were investigated and are presented in Table 17. Among the 13 vaccines, all include subserotype Ib/Ic, and only three vaccines were found to include suberotype Ia.

Table 17. Serotypes of inactivated vaccines containing *S. parauberis* antigens

Company name	Product name	Serotypes		
		Ia	Ib/Ic	II
㈜ 대성미생물연구소	대성 연쇄 피쉬백	-	+	-
㈜ 코미팜	프로백 더블에스	-	+	-
㈜ 고려비엔피	힘백 어질방 S-3 플러스	-	+	-
㈜ 하나윈	포세이돈-3	-	+	-
㈜ 대성미생물연구소	대성 연쇄 혼합 피쉬백	-	+	-
㈜ 하나윈	포세이돈-5	+	+	+
㈜ 코미팜	프로백 에드와드연쇄상구균 혼합백신	-	+	-
㈜ 중앙백신연구소	참신 연쇄 포커스	-	+	-
㈜ 코미팜	프로백TM 펜타	-	+	-
㈜ 고려비엔피	힘백 어질방 ES3 플러스 혼합 불활화 백신	-	+	+
㈜ 대성미생물연구소	대성 VES-2 피쉬백	-	+	-
㈜ 고려비앤피	윌로마린 S.E 4	+	+	-
㈜ 씨티씨백	씨티씨백 세균다자바 플러스	+	+	-

4. Discussion

Olive flounder, a representative species in domestic aquaculture, has firmly established itself as a vital industry, particularly in large-scale cultivation on Jeju Island. Among the diseases affecting olive flounder, streptococcosis of bacterial diseases, caused by *S. parauberis,* stands out as a significant affecting olive flounder. *S. parauberis* is a disease that infects not only saltwater fish but also freshwater fish, causing disease in various fish species (Kusuda et al., 1978; Baeck et al., 2006; Austin and Austin, 2007; Pradeep, 2016; Mishra et al., 2018). Domestic research on the serotype of *S. parauberis* is in progress, and as a result of monitoring the *S. parauberis* serotype isolated from Jeju Island farmed olive flounder, it was confirmed that the isolation pattern of serotypes changes depending on the year, which leads to the possibility of using a vaccine. reported that serotype monitoring is important (Kim et al., 2020). The purpose of this study was to analyze basic data on biochemical differences, antibiotic susceptibility mutations, and antibiotic resistance gene detection through *S. parauberis* serotype monitoring and provide basic data for vaccine development and serotype analysis.

From March 2019 to August 2023, a total of 382 isolates of *S. parauberis* were detected from diseased olive flounder in Jeju-si and Seogwipo-si fish farms. Among the three serotypes, subserotype Ia exhibited the highest detection rate at 73%, followed by subserotype Ib/Ic at 16%, and serotype II at 11%. Suberotype Ia consistently showed high detection rates each year (61% in 2019, 94% in 2020, 67% in 2021, 69% in 2022, and 80% in 2023). In contrast, subserotype Ib/Ic demonstrated a decreasing trend, with a detection rate. (32% in 2019, 4% in 2020, 13% in 2021, 0% in 2022, and 5% in 2023). Serotype II increased over the years, with detection rates (7% in 2019, 3% in 2020, 20% in 2021, 31% in 2022, and 15% in 2023). According to a previous study, the serotypes of *S. parauberis* isolated from Jeju Island farmed olive

flounder was observed from 2003 to 2020, and subserotype Ib/Ic were dominant from 2003 to 2010, and subserotype Ib/Ic were dominant after 2010. Subserotype Ia was dominant, and the detection rate of serotype II was confirmed to have increased after 2018 (Kim et al., 2020). In this study, as in the previous study, subserotype Ia was dominant from 2019 to 2023, and the detection rate of serotype II showed a tendency to increase, so the results are consistent with the previous results. During the period from 2019 to 2023, *S. parauberis* was collected, and results were organized based on the year and region to examine the detection patterns of serotypes.

In Jeju-si's Hangyeong area, *S. parauberis* was collected from 2023 onward, with no collection from 2019 to 2022. In Namwon area, Seogwipo-si, *S. parauberis* was collected in all years except 2021. In Pyoseon area, *S. parauberis* was collected in all years except 2020 and 2023. In 2019, *S. parauberis* strains were most detected in Seongsan, Daejeong, Namwon, Pyoseon, Gujwa, and Jocheon, with subserotype Ia consistently surpassing subserotype Ib/Ic and serotype II throughout the year. In 2020, the highest detection of *S. parauberis* strains occurred in Seongsan, Daejeong, Namwon, Gujwa, and Jocheon. In 2021, the highest detection of *S. parauberis* strains was in Seongsan, Daejeong, Gujwa, Jocheon, and Pyoseon. In 2022, the highest detection of *S. parauberis* strains occurred in Seongsan, Daejeong, Gujwa, Pyoseon, Jocheon, and Namwon. Subserotype Ia had the highest detection rate. In 2023, the highest detection of *S. parauberis* strains was in Daejeong, Hangyeong, Seongsan, Namwon, Jocheon, and Gujwa. Subserotype Ia had the highest detection rate. Throughout the experimental period, subserotype Ia was consistently the most detected across all olive flounder farms each year, and no clear monthly pattern in serotypes was identified. The variation in the number of olive flounder farms and collection areas each year led to differences in isolate numbers. After August 2023, no further collection of *S. parauberis* was conducted. Daejeong and Seongsan olive

flounder farms, where olive flounder collection takes place, are presumed to have a high level of isolate collection as there are more olive flounder farms used in experiments compared to other regions. For future serotype analysis based on regions, it is advisable to establish a standardized olive flounder farm count and conduct monitoring.

The results of serotype-specific biochemical analysis using the API 20 STREP kit no biochemical differences were observed among the serotypes. Previous studies using the API 20 STREP kit for biochemical identification of *Streptococcus* spp. yielded varied results, with only a few strains identified as *Streptococcus* spp., and low reliability for the remaining strains (Kim and Kim, 2003; Song et al., 2003; Lee et al 2007). Geographical location and physicochemical factors were found to influence biochemical characteristics (Nho et al., 2009). Despite identical strains, diverse results could arise from repeated experiments, and within the same species, the interpretation of API profiles could vary based on the experimenter's skill, leading to low reliability in the results (Cho et al., 2007b). Therefore, considering these limitations, it is concluded that for strain identification, utilizing genetic methods such as PCR, which are effective for the final identification of *S. parauberis*, is necessary.

In this study, experiments were conducted to compare the antibiotic susceptibility patterns of 19 antibiotics for *S. parauberis* serotype isolated from cultured olive flounder in Jeju Island in 2023. The antibiotic susceptibility results revealed that a significant number of isolates exhibited inhibition zones of 6mm or less for quinolone antibiotics such as oxolinic acid (89%), nalidixic acid (83%), flumequine (87%), sulfonamide antibiotics like sulfadiazine (82%), and aminoglycoside antibiotics including gentamycin (40%). For other antibiotics, strains with inhibition zones of 6mm or less were observed in less than 20-10% of cases. These findings align with previous research indicating high resistance rates of *S. parauberis* isolated from Jeju farmed olive flounder to oxolinic acid, nalidixic acid, and sulfadiazine

antibiotics, while showing low resistance rates to β-lactam antibiotics (Lee et al., 2018b).

The *tet*(M) gene has been identified as the predominant tetracycline resistance gene in many streptococci, including *S. pneumoniae* (Doherty et al., 2000) and *S. agalactiae* (Poyart et al., 2003). It is mainly associated with serotype II and is not detected in serotype I (Meng et al., 2009a; Meng et al., 2009b). The *tet*(S) gene is widely distributed in *Listeria* spp., and most isolates of *Enterococcus* spp. (Hammerum et al., 2004). Erythromycin resistance genes are closely related to tetracycline resistance genes, and most *erm* genes tend to be located on the chromosome and are associated with transposons, often co-transferred with *tet* genes. For instance, *erm*(A) gene is associated with *tet*(O) gene, and *erm*(B) gene is associated with *tet*(M) gene (Roberts et al., 1999; Chopra and Roberts, 2001; Martel et al., 2005; Brenciani et al., 2007). Contrary to previous findings, this study observed a tendency to possess both *tet*(S) and *erm*(B), particularly in 13 isolates of subserotype Ia. This aligns with previous research indicating the association of acquiring erythromycin and tetracycline resistance in *S. parauberis* isolates with *tet*(S) and *erm*(B) genes (Park et al., 2009; Woo et al., 2021; Lee et al., 2023). Furthermore, *tet*(S) and *erm*(B) resistance genes were detected in subserotype Ia, while *tet*(M) was identified in serotype II. This corresponds to research suggesting variations in the pattern of detected resistance genes based on serotypes (Woo et al., 2021; Lee et al., 2023). Additional research is needed to further confirm the association of tetracycline and erythromycin resistance genes in *S. parauberis* serotype.

From 2005 to 2009, subserotype Ib/Ic dominated (Kim et al., 2020). In 2015, differentiation of serotype I into Ia and Ib/Ic became possible due to capsular polysaccharides (Tu et al., 2015). Thus, all vaccines developed before 2015 could only distinguish between serotype I and II, including subserotype Ib/Ic prevalent at that time. However, since 2018, vaccines based on *S. parauberis*

serotype have been developed, with recent ones even including subserotype Ia. This study reveals the dominance of subserotype Ia and monitors an increasing detection rate of serotype II. Consequently, there is a need for new vaccines considering both subserotype Ia and serotype II.

Moreover, since the detection rate of *S. parauberis* serotype may change over time, it's essential to recognize the possibility that the antigen serotype of currently used vaccines may not match the latest prevalent serotype. Hence, pre-vaccination serotype and reliance on vaccines based on serotype patterns are deemed crucial. For these reasons, this study is anticipated to serve as a valuable reference for future *S. parauberis* serotype analyses.

5. Reference

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2019~2023년도 제주도내 양식 넙치(*Paralichthys olivaceus*)에서 분리된 *Streptococcus parauberis* 혈청형에 관한 연구

한지은

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요약

연쇄구균증을 일으키는 *S. parauberis*는 넙치 생산에 영향을 미치는 세균성 질병 중 하나이다. *S. parauberis*의 혈청형이 확인되면서 혈청형에 대한 연구가 진행되고 있다. 2020년 이후 제주도 양식 넙치에서 분리된 *S. parauberis* 혈청형에 대한 연구가 이루어지지 않아, 본 연구에서는 2019년부터 2023년까지 제주도 양식 넙치로부터 분리된 *S. parauberis* 혈청형에 대한 연구를 수행하였다. 2019년 3월부터 2023년 8월까지 제주도 양식장에서 질병에 감염된 넙치에서 분리한 382개의 *S. parauberis* 혈청형을 분석하였다. 2019년에는 155 균주, 2020년에는 80 균주, 2021년에는 30 균주, 2022년에는 32 균주, 그리고 2023년에는 85 균주가 분리되었으며, 이들 균주는 모두 3가지 혈청형으로 나뉘었다. 이 중에서 가장 많이 검출된 혈청형은 Ia로, 총 382 균주 중 279 균주 (73%)를 차지하였다. 혈청형 Ib/Ic는 61 균주 (16%), 혈청형 II는 42 균주 (11%)를 차지하였다. 혈청형 Ia은 연구 기간 동안 높은 검출률을 보였고, 혈청형 Ib/Ic는 연구 기간 동안 검출률이 감소하는 경향을 보였다. 반면, serotype II는 검출률이 증가하는 추세를 보였다. 혈청형에 따른 생화학적 차이를 확인하기 위해 API 20 STREP 키트를 사용하였으나, 혈청형에 따른 생화학적 차이는 확인되지 않았다. 항생제 감수성 실험 결과, quinolone, sulfonamide 및 aminoglycoside에 대한 높은 내성이 확인되었다.

항생제 내성 유전자 검출 결과, 혈청형에 따라 항생제 내성 유전자 검출 양상이 다른 것을 확인하였다. 혈청형 Ia에서는 *tet*(S) 및 *erm*(B) 내성 유전자가 검출되었고, 혈청형 II에서는 *tet*(M) 내성 유전자가 검출되었다. *S. parauberis* 혈청형을 포함하는 백신은 모두 혈청형 Ib/Ic를 포함하며, 혈청형 Ia를 포함하는 백신은 3종류로 확인되었다. 연구 기간 동안 혈청형 모니터링을 통해, 혈청형 Ia의 매년 높은 검출률을 가지며, 혈청형 II의 경우 검출률이 증가하는 경향을 확인하였다. *S. parauberis* 혈청형의 검출률은 연도에 따라 변화할 수 있으며, 현재 양식장에서 사용되는 백신의 항원 혈청형이 유행하는 혈청형과 일치하지 않을 수도 있기 때문에, 백신 접종 전 혈청형 모니터링을 기반으로 혈청형 검출 패턴에 따라 백신을 사용하는 것이 중요할 것으로 판단된다. 본 연구는 향후 *S. parauberis* 혈청형 모니터링 및 혈청형 분석에 대한 참고 자료가 될 것이다.

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