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Report on the Occurrence of *Perkinsus* sp. in the Manila Clams, *Ruditapes philippinarum* in Korea

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Five species of intertidal clams including *Ruditapes philippinarum, Tegillarca granosa, Solen strictus, Heteromacoma irus,* and *Coecella chinensis* were tested for the presence of the protozoan parasite, *Perkinsus* sp. using fluid thioglycollate medium (FTM) fortified with antibiotics and histological techniques. Each individual clam was placed in a test tube filled with 10 ml FTM, placed in totally dark place, and incubated over a week. After incubation the clam tissues were stained with Lugol's iodine solution and examined under a light microscope to find out any hypnospores of *Perkinsus* sp. in the tissues. Cross-sections of the clams were also embedded in paraffin, sliced to 3μm, and stained with Harry's hematoxylene and Picro eosine to observe the presence of tomont or trophozoites.

***Perkinsus* sp. were found in the tissues of *R. philippinarum* collected from Kangjin and Wando, along the south coast of Korea. However, *Perkinsus* sp. was not found in four other species of clams nor *R. philippinarum* collected from Kimnyong and Waido in Cheju. A size-dependent *Perkinsus* sp. infection was found in *R. philippinarum* collected from Kangjin and Wando · the clams smaller than 15 mm in shell width do not exhibit any *Perkinsus* sp. while other clams greater than 20mm in shell width exhibit almost 100% infection. To determine the number of *Perkinsus* sp. in the clams, FTM cultured clam tissues were digested with 2M NaOH solution and the number of hypnospores in the tube were counted. The number of hypnospores counted from the tissues indicated that each Manila clam contains 100,000 to 3,500,000 *Perkinsus* cells or 20,000 to 1,000,000 cells per gram tissue wet weight. The results of cell counts also suggests that such a high occurrence of *Perkinsus* sp. in the clam may cause mortality, as already reported from other studies of *Perkinsus* spp.**

Key words : *Perkinsus, Ruditapes philippinarum*, Bivalve parasites, FTM, Korea

Introduction

Phylum Apicomplexa contains a unique class, Perkinsea which has only one order, Perkinsida, one family, Perkinsidae, and one genus *Perkinsus* (Levin 1978). Up to now, four species of *Perkinsus* have been reported to science ; *P. marinus,* a typical protozoan parasite of the American oyster, *Crassostrea virginica* (Mackin et al., 1950 ; Ray, 1954 ; Andrews, 1955 ; Perkins, 1969 ; Levin 1978), *P. atlanticus* reported from Portuguese clams, *Ruditapes decussatus* (Azevedo et al., 1990), *P. olseni* from the gill and mantle tissue of the Australian blacklip abalone, *Haliotis ruber* (Lester and Davis,

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1981), and *P. karlssoni* from tissues of the bay scallop, *Argopecten irradians* (McGladdery et al., 1991). They are unicellular protozoans and are all known as parasites of marine mollusks, particularly oysters, littleneck clams, mya clams, and abalones. *P. marinus*, the originally described species are responsible for the mass mortality of the American oysters all along the south and east coast of the United States, especially in Louisiana and Chesapeake Bay (Burreson and Ragone Calvo, 1996). *Perkinsus* infection is known to be epidemic; the disease can be transmitted either via water column or other organisms such as parasitic snails or crabs. (Mackin, 1962 ; White et al., 1988 ; Dungan and Roberson, 1993). It is reported that moderate to heavy infection of *P. marinus* can disturb reproductive physiology of the *C. virginica* causing retarded reproductive maturity and lowered fecundity (Choi et al, 1989, 1991, 1993, 1994) *P. atlanticus* is also known to cause mass mortality of the Portuguese clams at a high level of infection (Azevedo et al., 1990).

Perkinsus spp exhibit a unique developmental cycle. They exhibit both motile and non-motile developmental stages. When inhabiting in the host tissues, they appear as a trophozoite, an uninucleate cell or tomont which is a multinucleate cyst containing numerous trophozoites formed by multiple binary fission of the trophozoites. Trophozoites are non-motile and very small in size, a typical ranges of 2 to 10μm in diameters (Perkins, 1969, 1996). Once free from host, they form another non-motile developmental stage, spherical hypnospores in an anaerobic condition within a few days. One of the remarkable feature of the hypnospores is its size enlargement; hypnospores of *P. marinus* in the American oysters have their size range of 20 to 200 μm in diameters, with a typical size range of 30 to 80 μm in diameter (Perkins, 1996). If the hypnospores are washed free of the FTM and incubated in sea water of 20 to 30‰, zoosporulation of the tomonts occurs. Biflagellated zoospores, the only motile developmental stage of *Perkinsus*, are then released from the zoosporulation of the tomonts. Subsequently the zoospores loose the flagellates and become immature trophozoites in the oyster tissues.

Ray (1954, 1966) has developed a simple method to detect *P. marinus* in the oyster tissues with fluid thioglycollate media (FTM) and Lugol's iodine. To detect the parasite, suspected animal tissues are placed in FTM tube and incubated over a week in a dark condition. During incubation, the trophozoites become hypnospores by remarkably expanding its size, without any reproduction. As a results, the hypnospores can be detected easily under microscopic field due to its size. After incubation the cultured tissues are stained with Lugol's iodine and examined under a microscope. If *P. marinus* present in the tissue, the tissues are then stained as dark blue or brown due to the presence of the hypnospores in the tissues. This FTM technique have been widely used in the diagnosis of *P. marinus* infection in the American oysters for the past four decades. The FTM technique is also acceptable in the study of other *Perkinsus species*, such as *P. atlanticus* in Portuguese clams and *P. olseni* in the blacklip abalone in Australia.

A study on the pathology of Manila clam *Ruditapes philippinarum* inhabiting along the south coast of Korea reports the presence of *Perkinsus* like organisms in the clams (Lee, personal communication). Although it is not proven, the *Perkinsus* disease may be responsible for the mass mortalities reported along the south coast of Korea for the past few years. Since *Perkinsus* infection is epidemic, most Manila clams occurring along the south coast of Korea may be already infected with *Perkinsus* like parasites. A survey on the occurrence of *Perkinsus species* have been conducted on five species of marine bivalves to investigate its prevalence, percent occurrence of the parasites, and the infection intensity. Here we report the observations made from the *Perkinsus* like organisms found in Manila clams, *R. philippinarum*.

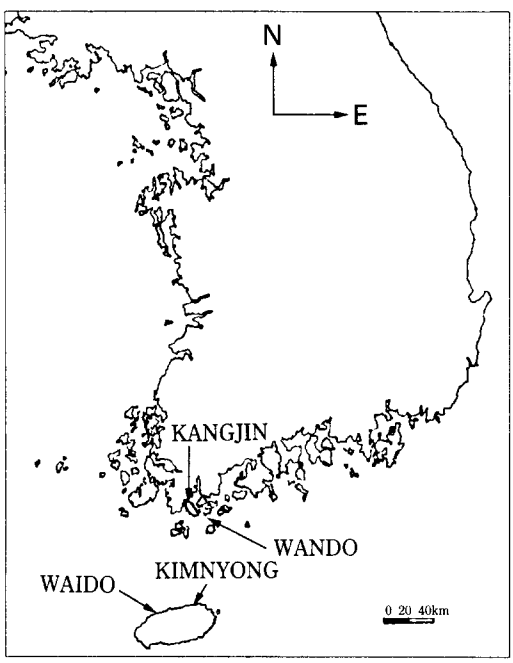

Fig. 1. Location of the sampling sites.

Materials and Methods

Sampling Area and Effort

A four intertidal areas with abundant species of marine bivalves were selected for collecting bivalve shells, Waido and Kimnyong in Cheju, Kangjin and Wando in the south coast (Fig. 1). The sampling sites in Kangjin and Wando exhibited well developed tidal flat, consisting of silty-mud sediments. Kimnyong in Cheju is a typical sand beach exhibiting fine or silty-sand. Waido is an estuarine environment with sandy-mud sediment.

Adult sizes of *R. philippinarum*, the Manila clam, *Coecella chinensis*, *Heteromacoma irus*, the macoma clam, *Tegillarca granosa*, the blood clam, and the razor clam, *Solen strictus* were collected depending upon sampling sites. To find size-dependent infection with *Perkinsus species* which might exist in the Manila clams as reported in the American oysters *C. virginica*, spats of the clams were also included in the analysis. Table 1 summarizes the number of species used and the number of individuals used in this study.

Fluid Thioglycollate Media (FTM) technique

The presence of *Perkinsus species* in suspected animals was determined with FTM methods which is a standard method in the diagnosis of *P. marinus* in the American oysters as well as in the Portuguese clams and the abalones. For developing hypnospores from suspected animal tissues, fluid thioglycollate media (FTM) was prepared

by mixing 20g of NaCl and 30g of dehydrated fluid thioglycollate media in 1 l distilled water. The mixture was then heated while stirring until the solution become transparent golden-yellow color. After cooling the solution was dispensed, 10 ml at a time, into 15 ml culture tubes which were subsequently autoclaved and sealed. The tubes were kept at dark until used. Thioglycollate maintains anaerobic conditions in the culture tube as well as providing needed nutrients and an appropriate osmotic environment. To prevent any bacterial activity during cultivation in FTM, chloromycetin and mycostatin mixture was prepared by adding 5 ml of distilled water into 1 g of chloromycetin (chloramphenicol), 10 ml of sterile water added to a 500,000 unit vial of mycostatin (nystatin), and transfer 2.5 ml of chloromycetin solution into the mycostatin vials. 50 μl of the antibiotic was added to each 10 ml FTM tube when used (Ray, 1966 ; Wilson-Ormond et al, 1993). 50 ml of FTM was also placed in a 100 ml glass bottle and 3 individuals of *R. philippinarum* were added to quantify the number of spores present in each individual.

Quantification of the Infection

After incubating in FTM over a week or more, the tissues were placed on a petri-dish and teased apart using sterile needles. The tissues were then flooded with Lugol's iodine solution and examined under a dissecting microscope. The Perkinsus hypnospores appeared as dark blue or brown color and due to some carbohydrate materials on the cell walls. Well developed lipid droplets are also observed on the surface of the spores. A modified Mackin's semi-quantitative scale was applied in the determination of infection intensity (Wilson-Ormond et al., 1993 ; Mackin, 1962). Table 2 illustrates the modified Mackin's scale applied in this study.

For counting real number of Perkinsus cells present in infected clams, the tissues incubated in FTM was first stained with Lugol's iodine solution, graded the infection intensity according to the modified Mackin's scale, placed in a 50 ml test tube, and centrifuged at 2000 rpm for 10 minutes to settle down the spores present in the media.

Table 1. Sampling sites and the bivalve species collected and analyzed for examining *Perkinsus species*. FTM : Fluid Thioglycollate Media (FTM) technique applied for analysis. Histology : histological slide is made from specimen for examining *Perkinsus species*. N : Number of individuals analyzed.

Date	Sampling Locality	Bivalve Species Collected(N)	Remarks
97/5/14	Kimnyong, Cheju	*Ruditapes philippinarum* (26) *Heteromacoma irus* (15) *Coecella chinensis* (8)	FTM Histology
97/5/14	Kangjin, Cheon-Nam	*Ruditapes philippinarum* (43) *Solen strictus* (12)	FTM Histology
97/7/23	Waido, Cheju	*Ruditapes philippinarum* (45)	FTM
97/6/23	Wando	*Ruditapes philippinarum* (30)	FTM Histology
97/6/23	Kangjin, Cheju	*Ruditapes philippinarum* (36) *Tegillarca granosa* (25)	FTM Histology

Table 2. A modified Mackin's scale used in this study for determining *Perkinsus* infection intensity

Infection intensity	Numerical code	Description
Heavy (H)	5	75 to 100% of tissue is covered with hypnospores
Moderate Heavy (M)	4	50 to 75% of the tissu is covered with hypnospores
Moderate (M)	3	25 to 50% of the tissue is covered with hypnospores
Light Moderate (LM)	2	>125 hypnospores occurs but less than 25% of the tissue is covered.
Light (L)	1	10 to 100 hypnospores present in the tissues
Very Light (VL)	0.5	Less than 10 hypnospores present
Negative (N)	0	No hypnospore present

FTM in the tubes was then removed and 30 ml of 2M sodium hydroxide (Choi et al., 1989, 1991) added onto the test tubes and incubated at 50℃ for 30 min. After incubation, the solution was removed and the same volume of 0.15M saline solution was added, spun at 2000 rpm for 10 min. This washing step was repeated for twice. Hypnospores were then resuspended in 10 ml saline solution and the number of spores in the solution was determined using a hemocytometer.

Histological Investigation

To examine the presence of *Perkinsus* species, usually as trophozoites, individual clams were fixed in Bouin's solution for 24 hr. After fixation, the samples were washed in running tab water for 12 hr to remove any residual picric acid in the Bouin's fixative. A cross section was made along the body of clam and dehydrated and embedded in paraffin. A 3 μm thick section was made using a microtome and fixed on a glass slide. After rehydration, the slides were stained with Harry's hematoxylene and counter stained with Picro eosin. The presence of Perkinsus species in the histological preparation was then examined using a light microscope.

Results and Discussion

Occurrence of Perkinsus like organisms

Investigation conducted on five species of marine bivalves using Ray's FTM method and histological analysis revealed that *Perkinsus* is not occurred in *S. strictus, T. granosa, H. irus, and C. chinensis.* FTM and histopathology conducted on *R. philippinarum* collected from two different sampling sites in Cheju also failed to detect *Perkinsus* like organisms in the tissues. However, trophozoites of *Perkinsus* sp. was observed on histological preparations of *R. philippinarum* collected from Kangjin and Wando. Multinucleate tomonts were common along the gill epithelium and around the visceral mass of *R. philippinarum* from Kangjin and Wando. FTM test performed on the clams collected in those areas also showed hypnospores in the examined tissues (Fig. 2 and Table 3). FTM results also indicated that *Perkinsus* species is not homogeneously distributed in the clams ; hypnospore formations is very distinct and abundant around the gills and visceral mass while the spores are rare around the foot and siphons(Fig. 3). The data indicate that *Perkinsus* species we found from the south coast of Korea must

Table 3. Results of the FTM test conducted on the five species marine bivalves, *Ruditapes philippinarum, Heteromacoma irus, Coecella chinensis, Solen strictus, and Tegillarca granosa*. N = number of individual analyzed, STD = Standard deviation, FTM results + = hypnospore present, − = hypnospore absent. Number of spores indicates the hypnospore cells in the FTM cultured tissues.

Dates	Bivalve Species	Locality	N	Shell Length ± STD (mm)	Tissue Weight ± STD (gram)	FTM results	Percent infection (%)	Number of spores/ Individual	Number of spores/gram tissue
5/14/97	*R. philippinarum*	Kimnyong Cheju	30	30.35 ± 1.62	−	−	0	0	0
5/14/97		Kangjin Chon-Nam	37	43.52 ± 4.45	4.23 ± 0.78	+	100	3,243,852 ± 5,783,409	695,433 ± 1,111,197
6/23/97		Kangjin Chon-Nam	28	27.53 ± 12.55	2.11 ± 1.76	+	61	697,321 ± 821,925	247,784 ± 301,433
6/23/97		Wando Chon-Nam	19	35.28 ± 5.79	2.92 ± 1.71	+	84	447,532 ± 590,218	121,179 ± 15,661
6/22/97		Waido Cheju	32	30.27 ± 5.11	1.38 ± 0.67	−	0	0	0
5/14/97	*H. irus*	Kimnyong Cheju	7	36.60 ± 5.43	2.71 ± 1.28	−	0	0	0
5/14/97	*C. chinensis*	Kimnyong Cheju	9	24.09 ± 2.71	−	−	0	0	0
5/14/97	*S. strictus*	Kangjin Chon-Nam	12	66.58 ± 4.07	−	−	0	0	0
6/23/97	*T. granosa*	Kimnyong Cheju	25	32.15 ± 4.31	2.91 ± 1.01	−	0	0	0

be host-specific parasite, as indicated by Azevedo et al. (1990).

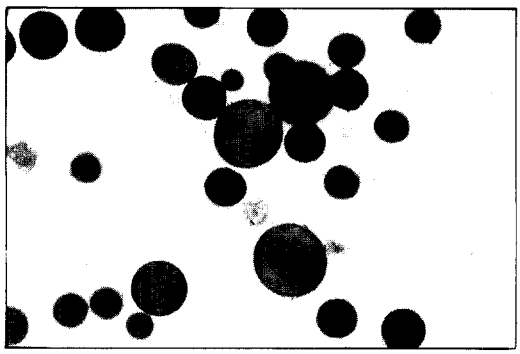

Fig. 2. Hypnospores of *Perkinsus species* found in *R. philippinarum* from Kangjin, Korea.

Infection Intensity and Prevalence

Among five different marine bivalves tested in this study, only *R. philippinarum* sampled in Kangjin and Wando exhibit *Perkinsus* species. The infection intensity of *Perkinsus* species is determined using two different methods. We use a modified Mackin's scale and the matching numerical code (Mackin, 1962, Wilson-Ormond et al., 19 93). The prevalence, percent infection of the individuals analyzed, is 61% among the clam collected from Kangjin (Table 4). From a total of 28 *R. philippinarum* analyzed, 17 clams are infected with *Perkinsus* and the infection intensity varies from moderate to heavy. The remaining 11 clams are found to be uninfected. It is noticed that uninfected clams are very small, mostly spat size while infected clams are adult size with shell length over 20mm. It is believed that the clams greater than 20mm in shell length are at least a year old or more and most clams infected with Perkinsus are two to four year old (Chung et al., 1994). Therefore, a size dependent *Perkinsus* infection is obvious among *R. philippinarum*. Table 5 summarizes the FTM test results of *R. philippinarum* collected from Wando. Only three clams out of 19 analyzed are uninfected and their size is much smaller than other infected clams, supporting the size-dependent *Perkinsus* infection as observed in the clams from Kangjin. The size-dependent infection is well known in *P. marinus* in the American oyster, *C. virginica*. Numerous researches have reported that adult oysters are much more susceptible than the smaller or juvenile oysters (Ray, 1966, 1996 ; Andrews, 1996 ; Andrews and Hewatt, 1957). However, size-dependent infection in other species of *Perkinsus* (i.e., *P. atlanticus* in Portuguese clam and *P. olseni* in blacklip abalone) has not been reported yet.

The FTM cultivated clams are digested with 2M sodium hydroxide and the number of hypnospores presented in the tissues are counted in this study (Choi et al., 1989, 1991 ; Bushek et al., 1994 ; Fisher and Oliver, 1996). Table 4 shows that the individual clams

Fig. 3. *R. philippinarum* from Kangjin showing hypnospores of *Perkinsus* sp. Black stains are hypnospores of *P.* sp. TIssues are cultivated in FTM for 7 days and stained with Lugol's iodine.

Table 4. FTM results of *R. philippinarum* collected from Kangjin on June 23, 1997. L=shell length in mm, TWWT=total tissue wet weight in gram, N. spores/Unit Wt=number of hypnospores per gram tissue wet weight, N. code=Mackin's scale expressed as a numerical code, see Table 2.

No	L (mm)	TWWT (g)	N.spores/ Individual	N spores/ Unit Wt (g)	Mackin's Code	N. code
1	17.70	0.260	0	0	N	0
2	12.20	0.089	0	0	N	0
3	9.35	0.042	0	0	N	0
4	10.70	0.048	0	0	N	0
5	10.70	0.058	0	0	N	0
6	11.90	0.083	0	0	N	0
7	12.30	0.075	0	0	N	0
8	10.50	0.059	0	0	N	0
9	10.30	0.056	0	0	N	0
10	10.75	0.066	0	0	N	0
11	38.25	3.564	1,162,500	326,178	H	5
12	40.25	4.302	1,715,625	398,797	H	5
13	40.15	4.617	1,050,000	227,420	M	3
14	40.10	3.988	496,875	124,593	M	3
15	39.10	3.841	778,125	202,584	H	5
16	37.65	2.170	1,415,625	652,362	H	5
17	36.70	3.811	843.750	221,399	H	5
18	36.00	3.167	3,675,000	1,160,404	H	5
19	37.60	3.323	1,340,625	403,438	H	5
20	30.50	2.081	440,625	211,737	H	5
21	41.85	5.103	806,250	157,995	H	5
22	37.60	3.435	1,096,875	319,323	M	3
23	29.20	1.737	900,000	518,135	H	5
24	31.10	1.799	1,425,000	792,107	H	5
25	30.55	1.682	700,000	416,171	H	5
26	31.50	1.947	1,471,875	755,971	H	5
27	36.10	3.515	0	0	N	0
28	40.20	4.179	206,250	49,354	M	3
Mean	27.53	2.110	697,321	247,785		2.75

collected from Kangjin contain 200,000 to 1,400,000 *Perkinsus* hypnospores or 49,000 to 1,160,000 spores per gram wet tissue. The number of spores in the individual clams collected from Wando varies from 37,000 to 1,575,000 or 25,000 to 539,000 per gram tissues (Table 5). Since the number of clams analyzed using FTM is limited in this study, the exact correlation between the Mackin's numerical scale and the real number of spores present in each category of infection could not be determined. No comparable data for the number of *Perkinsus species* present in the infected *R. philippinarum* are present yet. Choi et al. (1989) have developed a technique to quantify the number of spores in FTM cultured oyster tissues using 2M NaOH and quantified the Mackin's numerical scale as a number of hypnospores. They have reported that the American oysters moderately to heavily infected with *P. marinus* may contain 100,000 to

Table 5. FTM results of *R. philippinarum* collected from Wando on June 23, 1997. L=shell length in mm, TWWT=total tissue wet weight in gram, N. code=Mackin's scale expressed as a numerical code, see Table 2.

No	SL (mm)	TWWT (g)	N.spores/ Individual	N Spores/g TWWT	Mackin's Code	N. code
1	47.80	6.67	881,250	132,141	M	3
2	43.20	4.42	112,500	25,458	L	1
3	37.60	3.80	1,575,000	414,038	MH	4
4	33.10	1.64	37.500	22,866	L	1
5	29.00	1.40	37,500	26,862	L	1
6	41.50	5.26	825,000	156,993	LM	2
7	35.90	2.88	440,625	153,048	LM	2
8	44.60	6.68	1,368,750	205,025	H	5
9	34.90	2.56	46,875	18,289	LM	2
10	37.35	2.28	140,625	61,786	M	3
11	32.20	1.87	450,000	240,642	LM	2
12	39.10	3.57	1,921,875	539,095	H	5
13	27.50	1.31	0	0	N	0
14	32.45	2.49	271,875	109,406	M	3
15	29.70	1.36	0	0	N	0
16	33.70	2.03	206,250	101,551	H	5
17	30.60	2.05	131.250	63,900	H	6
18	29.20	1.48	0	0	N	0
19	30.90	1.80	56,250	31,302	L	1
Mean	35.28	2.92	447,532	121,179		1.77

over 1,000,000 hypnospores per wet tissue weight.

Effects of Perkinsus Infection

A number of researches have been conducted on effect of *P. marinus* on the growth and physiology of the American oysters (Ray, 1996 ; Andrews, 1996). At a low infection level of *P. marinus* does not disturb the growth or reproduction of the oysters. However, as infection progresses and the oysters become heavily infected with *P. marinus*, the infected oysters become gaping and exhibit retarded growth and reproduction (Mackin, 1962 ; Choi et. al, 1989, 1993, 1994). *P. olseni* also has been associated with mortality in blacklip abalone, *Haliotis ruber* in Australia (Lester, 1980 ; Goggin and Lester, 1987). Azevedo (1989) also has reported a mass mortality of *Tapes decussatus* associated with *P. atlanticus*. One of the critical effect of the *Perkinsus* disease is a steady drain of the available energy from the host animals. Choi et al. (1989) calculated that the energy budget of the oyster become negative when the oysters are in moderate to heavy infection level.

Compare to the studies done on *P. marinus*, relatively few researches have been conducted on ecological aspects of *Perkinsus* disease in the Manila clams. It could be possible that some cases of mass mortality occurred in the Manila clams along the south coast of Korea may be associated with *Perkinsus species* found in this study, although any histopathological stdudies have

been made yet. Since *R. philippinarum* is one of the most important marine bivalve species in shellfish fisheries in Korea, a nation-wide survey program should be started to understand the prevalence and infection intensity of *Perkinsus species* to manage and protect the wild population of *R. philippinarum.*

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