TABLE 1: *Mycoplasma* species isolated from bulk tank milk and quarter milk samples

Herd no.	Number of cows	*Mycoplasma* species isolated from bulk tank milk	*Mycoplasma* species isolated from quarter milk samples (number of infected cows)		Total number of infected cows
			Single infection	Multiple infection	
1	45	*M alkalescense*	*M alkalescense* (2)		2
2	55	*M californicum*	*M californicum* (1)		1
3	56	*M bovis*	*M bovis* (2)		2
4	60	*M arginini*	*M arginini* (2)		2
5	61	*M bovis/ M arginini*	*M bovis* (3)	*M bovis+M arginini* (2)	5
6	72	*M alkalescense*	*M alkalescense* (2)		2
7	72	*M bovis*	*M bovis* (6)		6
8	80	*M californicum/M bovigenitalium*	*M californicum* (3)	*M californicum+M bovigenitalium* (2)	5
9	92	*M bovis*	*M bovis* (5)		5
10	105	*M bovis/ M. canadense*	*M bovis* (1)/*M canadense* (3)	*M bovis+M canadense* (2)	6
11	155	*M bovigenitalium*	*M bovigenitalium* (4)		4
12	173	*M bovis*	*M bovis* (6)		6
13	182	*M californicum*	*M californicum* (3)		3
14	195	*M bovis/M. bovigenitalium*	*M bovis* (2)/*M bovigenitalium* (4)	*M bovis+M bovigenitalium* (2)	8
15	201	*M canadense*	*M canadense* (1)		1
16	217	*M arginini*	*M arginini* (2)		2
17	215	*M bovis*	*M bovis* (7)		7
18	230	*M adleri*	*M adleri* (4)		4
19	237	*M bovis/M canadense/ M arginini*	*M bovis* (7)/*M canadense* (1)	*M bovis+M canadense+M arginini* (2)	10
20	241	*M bovis/M californicum/ M bovigenitalium*	*M bovis* (4)/*M californicum* (2)	*M bovis + M californicum + M bovigenitalium* (1)	7
21	274	*M bovis/M californicum*	*M bovis* (9)/*M californicum* (1)	*M bovis+M californicum* (2)	12
22	328	*M californicum/M canadense*	*M californicum* (4)/*M canadense* (1)	*M californicum+M canadense* (1)	6

TABLE 2: *Mycoplasma* species isolated from quarter milk samples, and their relationship to geometric somatic cell count (SCC) and clinical signs

Mycoplasma species	Total		Geometric SCC ≥200 000 cells/ml		
	Number of *Mycoplasma* species isolated from cows	Number of *Mycoplasma* species isolated from quarters	Number of quarters (%)	Somatic cell count (geometric mean±SD, $\times 10^3$/ml)	Clinical signs in quarters (%)
Single infection					
M bovis	52	124	115 (93)	982±421[a]	112 (97)
M californicum	14	32	28 (88)	853±294[a]	26 (93)
M bovigenitalium	8	10	6 (60)	622±321[b]	6 (100)
M canadense	6	6	4 (67)	685±313[b]	4 (100)
M arginini	4	4	2 (50)	656	1 (50)
M alkalescense	4	4	1 (25)	524	0
M adleri	4	4	1 (25)	463	0
Multiple infection					
M bovis+M californicum	2	5	5 (100)	2263±884[c]	5 (100)
M bovis+M bovigenitalium	2	4	4 (100)	1951±812[c]	4 (100)
M bovis+M canadense	2	3	3 (100)	2066±623[c]	3 (100)
M bovis+M arginini	2	3	3 (100)	1923±781[c]	3 (100)
M californicum+M bovigenitalium	2	3	3 (100)	1965±656[c]	3 (100)
M californicum+M canadense	1	2	2 (100)	1288	2 (100)
M bovis+M californicum+M bovigenitalium	1	2	2 (100)	1382	2 (100)
M bovis+M canadense+M arginini	2	2	2 (100)	2122	2 (100)
Total	**106**	**208**	**181 (87)**		**138 (66)**

Different superscripts indicate a statistically different SCC
a–c, b, c: $p < 0.01$, a, b: $p < 0.05$

response in quarters due to synergistic effects, and it might be related to abnormal SCC and clinical signs. Published reports on synergism between two or three *Mycoplasma* species are rare and, thus, synergism between these *Mycoplasma* species in bovine intramammary infections should be clarified.

References

BUSHNELL, R. B. (1984) Mycoplasma mastitis. *Veterinary Clinics of North America: Large Animal Practice* **6**, 301–312

HEWICKER-TRAUTWEIN, M., FELDMANN, M., KEHLER, W., SCHMIDT, R., THIEDE, S., SEELIGER, F., WOHLSEIN, P., BALL, H. J., BUCHENAU, I., SPERGSER, J. & ROSENGARTEN, R. (2002) Outbreak of pneumonia and arthritis in beef calves associated with *Mycoplasma bovis* and *Mycoplasma californicum*. *Veterinary Record* **151**, 699–703

HIGUCHI, H., IWANO, H., GONDAIRA, S., KAWAI, K. & NAGAHATA, H. (2011a) Prevalence of *Mycoplasma* species in bulk tank milk in Japan. *Veterinary Record* **22**, 169

HIGUCHI, H., IWANO, H., KAWAI, K., OHTA, T., OBAYASHI, T., HIROSE, K., YOKOTA, H., TAMURA, Y. & NAGAHATA, H. (2011b) A simplified PCR assay for fast and easy mycoplasma mastitis screening in dairy cattle. *Journal of Veterinary Science* **12**, 191–193

INFANTE-MARTINEZ, F., AGUADO, J. & EDUARD-JASPER, D. (1999) Mastitis outbreak due to *Mycoplasma californicum* and *Mycoplasma canadense* in a commercial dairy herd in the state of Jalisco, México. *Revista Latinoamericana de Microbiologia* **41**, 117–120

JASPER, D. E. (1982) The role of mycoplasma in bovine mastitis. *Journal of American Veterinary Medical Association* **181**, 158–162

JASPER, D. E., DELLINGER, M. S., ROLLINS, M. H. & HANSON, M. S. (1979) Prevalence of *Mycoplasma* bovine mastitis in California. *American Journal of Veterinary Research* **40**, 1043–1047

KAUF, A. C., ROSENBUSCH, R. F., PAAPE, M. J. & BANNERMAN, D. D. (2007) Innate immune response to intramammary *Mycoplasma bovis* infection. *Journal of Dairy Science* **90**, 336–348

KIRK, J. H. & LAUERMAN, L. H. (1994) Mycoplasma mastitis in dairy cows. *Veterinarian* **16**, 541-551

NICHOLAS, R. A. & AYLING, R. D. (2003) *Mycoplasma bovis*: disease, diagnosis, and control. *Research in Veterinary Science* **74**, 105–112

REILLY, G. A., KENNEDY, S., McALISKEY, M. & BALL, H. J. (1993) The pathology of experimental *Mycoplasma californicum* mastitis in ewes. *Journal of Comparative Pathology* **109**, 155–161

SCHEPERS, A. J., LAM, T. J., SCHUKKEN, Y. H., WILMINK, J. B. & HANEKAMP, W. J. (1997) Estimation of variance components for somatic cell counts to determine thresholds for uninfected quarters. *Journal of Dairy Science* **80**, 1833–1840

SCHUKKEN, Y. H., WILSON, D. J., WELCOME, F., GARRISON-TIKOFSKY, L. & GONZALEZ, R. N. (2003) Monitoring udder health and milk quality using somatic cell counts. *Veterinary Research* **34**, 579–596

Veterinary Record

Mycoplasma species isolated from intramammary infection of Japanese dairy cows

H. Higuchi, S. Gondaira, H. Iwano, et al.

Veterinary Record 2013 172: 557 originally published online March 1, 2013
doi: 10.1136/vr.101228

Biocontrol Science, 2013, Vol. 18, No. 4, 193−197

Original

ELISA Detection of *Kudoa septempunctata* in Raw *Paralichthys olivaceus* (Olive Flounder) using a Chicken Anti-*Kudoa* Antiserum

YUTAKA KIKUCHI[1]*, TAKAHIRO OHNISHI[1], HIROKO FURUSAWA[1], TAKAO KAWAI[2], YUTAKA FUKUDA[3], HIROSHI YOKOYAMA[4], AND YOSHIKO SUGITA-KONISHI[1,5]

[1]*Division of Microbiology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[2]*Division of Bacteriology, Osaka Prefectural Institute of Public Health, 1-3-69 Nakamichi, Higashinari-ku, Osaka 537-0025, Japan*
[3]*Forestry and Fisheries Research, Oita Prefectural Agriculture, 194-6 Kamiura-Tsuiura, Saiki, Oita 879-2602, Japan*
[4]*Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*
[5]*Present address: Laboratory of Food Hygiene, Department of Food and Life Science, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan*

Received 28 January, 2013/Accepted 17 June, 2013

***Kudoa septempunctata* is the causative agent of a foodborne disease associated with the consumption of raw *Paralichthys olivaceus* (olive flounder). Chickens were used to establish specific antibodies against *K. septempunctata* spores. A specific antiserum, CS#3, raised against sonicated spores, also recognized intact spores. The CS#3 antiserum showed high titers for sonicated and intact *K. septempunctata* spores and was suitable for both ELISA and immunohistochemical staining. Using homogenated raw olive flounder meat, the ELISA system detected more than 5.0×10^5 spores in 1 g of tissue, which was consistent with the number determined by microscopic examination. The preparation of rapid detection kits for *K. septempunctata* spores in *P. olivaceus* muscle tissue using immunochromatography with CS#3 antiserum should be useful for preventing the foodborne disease in the field.**

Key words : *Kudoa septempunctata* / *Paralichthys olivaceus* / Chicken serum / ELISA / Immunohistochemical staining.

INTRODUCTION

Outbreaks of a foodborne disease associated with the consumption of raw *Paralichthys olivaceus* (olive flounder) have increased within the last decade in Japan, and there are now an average of more than 100 cases per year (Kawai et al, 2012). The causative agent of this novel foodborne disease is *Kudoa septempunctata* (Kawai et al., 2012). *K. septempunctata* spores are composed of six or seven shell valves and polar capsules (Matsukane et al., 2010). At present, two methods are used to detect *K. septempunctata* in *P. olivaceus*: microscopic observation (Matsukane et al., 2010) and PCR analysis (Kawai et al., 2012). However, these methods are not suitable for multiple samples and high-throughput screening. Therefore, a chicken anti-*K. septempunctata* antiserum was developed that would support the identification of spores in olive flounder meat by ELISA and immunohistochemical staining.

*Corresponding author. Tel: +81-3-3700-1141, Fax: +81-3-3707-6950, E-mail : kikuchi(a)nihs.go.jp

MATERIALS AND METHODS

Preparation of *Kudoa* spores

P. olivaceus meat infected with *K. septempunctata* was obtained from the fish farm at the Forestry and Fisheries Research, Oita Prefectural Agriculture. A portion was used for purification of *K. septempunctata*, while the rest was used to test the ELISA assay. The spores were purified from the trunk muscles of olive flounders using Percoll density gradient centrifugation (Chase et al., 2001). The purified intact spores were suspended in 50% glycerin-phosphate-buffered saline (PBS) and sonicated using a Sonifier Cell Disruptor 185 (power 1.5, 60 sec, 4°C) (Branson Ultrasonics, Danbury, CT, USA). The BCA protein assay was used to determine protein concentration (Thermo Scientific, Rockford, IL, USA). One milligram (wet weight) of sonicated *K. septempunctata* spores was equivalent to 3.2 μg of bovine serum albumin (BSA).

Microscopic examination of *Kudoa* spores

The number of *Kudoa* spores in raw fish meat was counted using a hemocytometer (Kawai et al., 2012). A 1-g portion of *P. olivaceus* muscle tissue was suspended in 4 mL of phosphate-buffered saline (PBS) and sieved through a 200-μm nylon sheet. The extract was passed through a 100-μm cell strainer (BD) and centrifuged at 1500 *g* for 15 min at 4°C. The pellet containing *Kudoa* spores was suspended in 1 mL of PBS, and any spores that were present were counted using a hemocytometer.

Strains

K. iwatai was obtained from the muscle tissues of *Acanthopagrus latus* obtained from Oita Prefecture. *P. olivaceus* muscle tissues infected with *K. lateolabralis* and *K. thyrsites* were obtained from Oita and Ehime Prefectures, respectively.

Preparation of Antiserum

Chicken anti-*Kudoa* antiserum was raised against sonicated *K. septempunctata* spores as previously described (Matsuda et al., 1999). Briefly, 1.5 mg (wet weight) of sonicated *K. septempunctata* spores in 150 μl of 50% glycerin-PBS combined with an equal volume of alum solution was used as an immunogen. Four 12-week-old White Leghorn chickens (Line-M 6, ♀) (Nippon Institute for Biological Science, Japan) were immunized with intramuscular injections of 1.5 mg of the immunogen at 14- to 28-day intervals. ELISA-positive antiserum against *K. septempunctata* spores was obtained from one of the Leghorns and labeled CS#3.

Enzyme-linked immunosorbent assay (ELISA) and competitive ELISA

ELISAs were performed as previously described (Kikuchi et al., 1991). A dilution buffer of 0.1% casein-PBS (C-PBS) was used throughout this study, and all washes were completed with 0.05% Tween 20-PBS. Briefly, 50 μl of sonicated *Kudoa* spores [40 μg (wet weight)/ml] were added to a 96-well flat-bottomed plate. The plates were centrifuged (1,600 *g*) at 4°C for 15 min and incubated at 4°C overnight. Before the assay, the wells were blocked with C-PBS for 30 min and washed three times. Serially diluted antiserum was added to the wells and incubated at room temperature for 60 min. After washing, β-galactosidase-conjugated goat anti-chicken IgG antibody (American Qualex Antibodies, San Clemente, CA, USA) in C-PBS was added to each well and incubated for 60 min. After washing, 4-methylumbelliferyl-β-D-galactoside substrate in 0.1 M phosphate buffer-1.5 mM $MgCl_2$-0.1 M 2-mercaptoethanol (pH 7.2) was added to the wells and incubated at 37°C for 60 min. Enzyme activity was determined by fluorescence intensity using a Arvo-SX (Perkin Elmer, Waltham, MA, USA). The titer of antiserum against *K. septempunctata* was defined as the reciprocal of the dilution that yielded half maximum binding.

The antiserum was then tested for use against *K. septempunctata* spores in olive flounder. Briefly, 100 mg of the trunk muscles of the olive flounder and 1.0 ml of 10% glucose-PBS were homogenated using a Multi-Beads Shocker (Yasui Kikai, Japan). After centrifugation (20,000 *g*) for 5 min, the supernatants (50 μl) were added to the wells. Plates were centrifuged (1,600 *g*) at 4°C for 15 min and incubated overnight at 4°C. The plates were treated as described above. The results confirmed that the antiserum recognized coated *K. septempunctata* spores as the solid-phase antigen.

In competitive ELISA, appropriately diluted inhibitors or samples were added to antigen-coated wells and incubated, in a total volume of 50 μl, with the CS#3 serum diluted to a concentration giving half-maximal binding. Wells were treated as described above, and the ratio of fluorescence intensity in the presence of an inhibitor to that in the absence of the inhibitor (B/Bo) was determined. The IC_{50} value is defined as the concentration of inhibitor giving 50% inhibition.

Immunostaining

Kudoa spores were immunostained using the chicken anti-*Kudoa* antiserum CS#3 with a modified peroxidase protocol (Sumi et al., 1993). After deparaffination and hydration of the tissue sections, endogenous peroxidase activity was removed. The sections were then

overlaid with goat serum (Dako, Glostrup, Denmark) for 30 min and CS#3 (1:200) for 50 min at room temperature. Normal chicken serum (Nippon Biotest Laboratories, Tokyo, Japan) was used as an immunostaining control. After washing, HRP-conjugated goat anti-chicken IgG (1:400) (American Qualex Antibodies, San Clemente, CA, USA) was allowed to react with the sections for 30 min at room temperature. The sections were stained with 0.02% 3,3'-diaminobenzidine-0.003% H_2O_2-50 mM Tris-HCl (pH 7.4) for 10 min at room temperature, followed by washing with running tap-water. The sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted.

Olive flounder was used as an immunohistochemical control. Sections prepared from tissues infected with *K. septempunctata* or without infection were stained in the same manner as described above.

RESULTS

Production and characterization of the chicken anti-*Kudoa* antiserum

The reactivities of the chicken anti-*Kudoa* antiserum CS#3 were investigated after it was obtained following immunization with sonicated *K. septempunctata* spores. CS#3 recognized both sonicated and intact spores as solid-phase antigens (FIG. 1). The titer of CS#3 antiserum against sonicated spores was 18,000 and 7,700 against intact spores (FIG. 1).

Cross-reactivity of the serum with various *Kudoa* spores was determined by competitive ELISA. The IC_{50} value of CS#3 was 70 ng (BSA equivalent)/ml for sonicated *K. septempunctata* spores and 200 ng (BSA equivalent)/ml for intact spores (FIG. 2A). The assay quantified sonicated *K. septempunctata* spores within the range of 0.5-10 ng (BSA equivalent)/50 μl/well. In contrast, CS#3 did not recognize another species of *Kudoa* spores, *K. iwatai*. The IC_{50} values of CS#3 antiserum for sonicated *K. iwatai* spores purified from the trunk muscles of *Pagrus major* was greater than 15 μg (BSA equivalent)/ml under the conditions used (FIG. 2B).

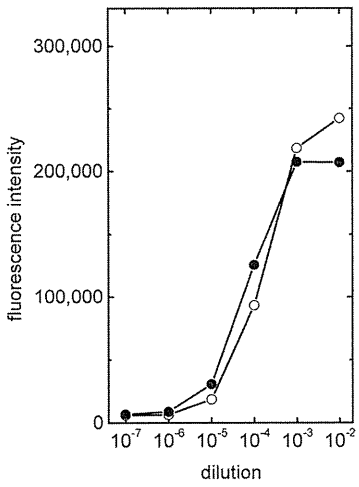

FIG. 1. ELISA using *K. septempunctata* spores as solid-phase antigens. The wells were coated with sonicated (●) or intact (○) *K. septempunctata* spores, and the ELISA was performed with serial dilutions of the chicken anti-*K. septempunctata* serum CS#3. The panel shows a plot of fluorescence intensity versus serum dilution. The results are shown as the means from two wells.

Immunohistochemical staining of *K. septempunctata* spores in the trunk muscles of *P. olivaceus* is shown in FIG. 3. Hematoxylin and eosin staining (HE staining) revealed the characteristic pseudocysts of *Kudoa* spp. in a section of muscle tissue infected with *K. septempunctata* (FIG. 3A). In another section of muscle tissue infected with *K. septempunctata*, immunostaining for *K. septempunctata* reaction products localized in the same manner as HE staining (FIG. 3B). The CS#3 antiserum also recognized *K. lateolabralis* (C) and *K. thyrsites* (D) spores in tissue sections. Tissue sections from the control trunk muscles of *P. olivaceus* showed no positive reaction products following immunostaining for *K. septempunctata* (FIG. 3E).

To validate the ELISA system, *K. septempunctata* spore content in the trunk muscles of the olive flounder was determined by both ELISA and microscopic exami-

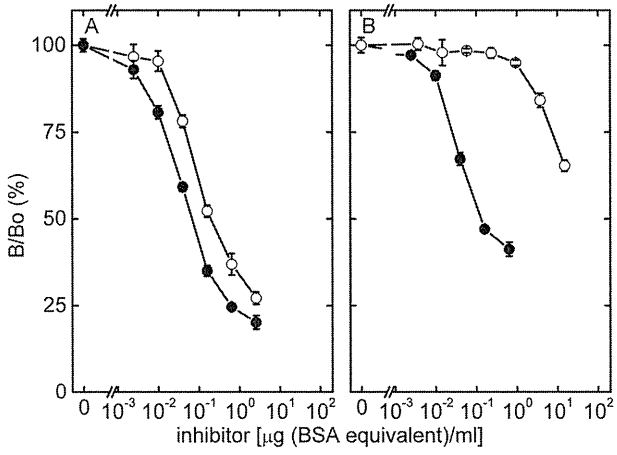

FIG. 2. Competitive ELISA using the chicken anti-*K. septempunctata* serum CS#3. A. Inhibition of sonicated *K. septempunctata* spores (solid-phase antigen) by sonicated (●) or intact (○) *K. septempunctata* spores. B. Inhibition of sonicated *K. septempunctata* spores (solid-phase antigen) by sonicated *K. septempunctata* (●) or *K. iwatai* (○) spores. The ratio of the signal of bound sonicated *K. septempunctata* spores in the presence of inhibitors to the signal in the absence of inhibitors (B/Bo) was plotted against inhibitor concentrations. The results are shown as the means ± SD from triplicate wells.

nation. Using homogenated raw olive flounder meat, the ELISA system found more than 5.0×10^5 spores in 1 g of tissue (FIG. 4). The number of spores determined by ELISA was consistent with that obtained by microscopic examination.

DISCUSSION

Several monoclonal antibodies (mAbs) have been raised against *K. thyrsites* spores (Chase et al., 2001). They recognize polar capsules, polar filaments and spore surface antigens. However, hybridoma techniques were not able to produce mAbs with sufficient specificity and affinity to detect *K. septempunctata* spores. Attempts to raise rabbit polyclonal antisera against *K. septempunctata* spores have also failed. It is well known that the chicken is a useful animal for the development of the specific antibodies against the mammalian conserved proteins. In this study, chickens were then used to acquire specific antibodies against *Kudoa* spp.

From one of four immunized Leghorn chickens, an ELISA-positive anti-*K. septempunctata* serum (CS#3) was obtained. The CS#3 antiserum was suitable for both ELISA and immunohistochemical staining, and was adaptable for detection of *K. septempunctata* sporoplasm inversion of Caco-2 cells by indirect immunofluorescence (Ohnishi et al, 2013).

The CS#3 antiserum raised against sonicated spores also recognized intact spores. Consequently, this competitive ELISA system is applicable for specific detection of both sonicated and intact spores. The ratio of IC_{50} values of CS#3 antiserum for sonicated and intact *K. septempunctata* spores (intact spores/sonicated spores) was 2.86 (FIG. 2A). It is possible the system tends to underestimate the content of *Kudoa* spores in intact samples. Thus, we homogenized raw materials using a Multi-Beads Shocker to estimate *Kudoa* spore content before using the ELISA system.

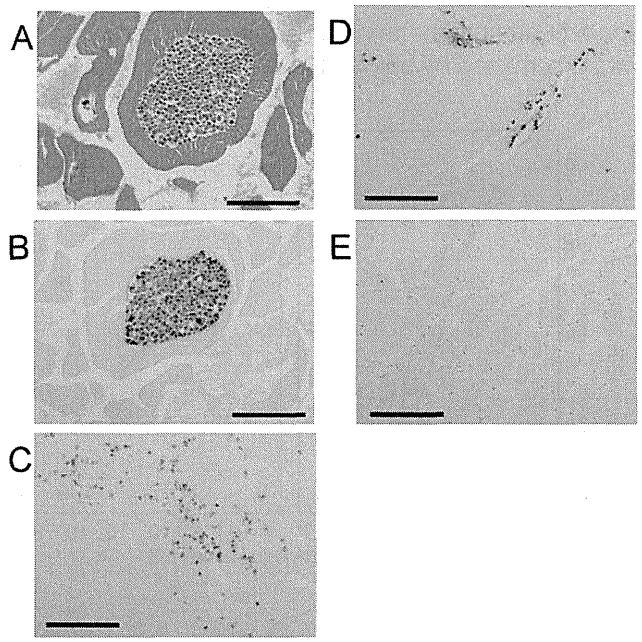

FIG. 3. Immunostaining of *Kudoa* spores using the chicken anti-*K. septempunctata* serum. *P. olivaceus* muscle tissues infected with *K. septempunctata* (A, B), *K. lateolabralis* (C) or *K. thyrsites* (D) were subjected to hematoxylin and eosin-staining (A) or immunohistochemical staining with CS#3 (B-D) as described under MATERIALS AND METHODS. *P. olivaceus* muscle tissue without *Kudoa* spp infection was used as an immunohistochemical control (E). Scale bars are 100 μm.

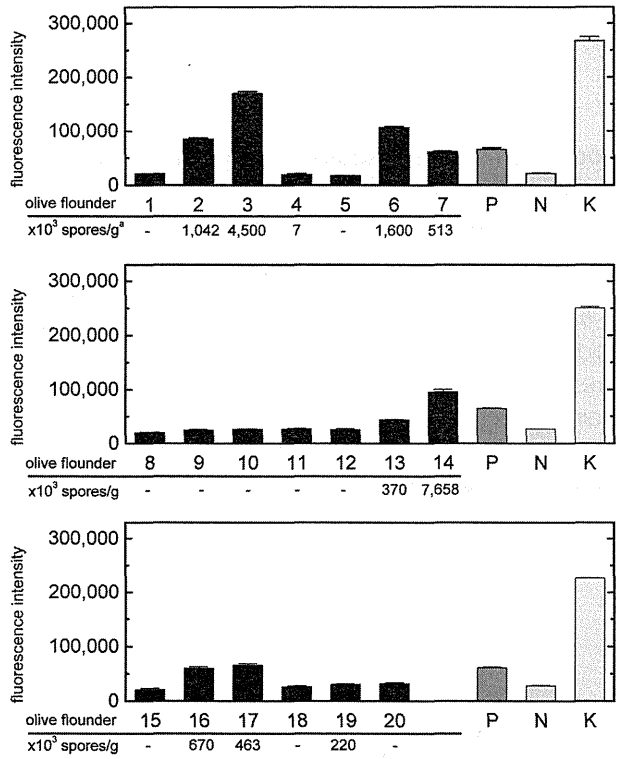

FIG. 4. Correlation between *K. septempunctata* spore content in raw olive flounder meat determined by ELISA and microscopic examination. Homogenated raw olive flounder meat (1 to 20; black bars) was coated onto the wells. Homogenated olive flounder meat infected with (positive control, P; magenta bars) or without (negative control, N; cyan bars) *K. septempunctata* spores, and sonicated *K. septempunctata* spores (K; yellow bars) were also coated onto wells of each plate. The coated plates were then used for ELISAs with CS#3 antiserum as described under MATERIALS AND METHODS. The panels show fluorescence intensity (bars on the vertical axis) versus spore contents as determined by microscopic examination (horizontal axis). Each panel shown as distinct ELISA-plate. Bars represent means ± SD (n=6). [a]Counts of *K. septempunctata* spores in tissues by hemocytometer; -, not detected.

To confirm the efficiency of the ELISA for detection of *K. septempunctata* spores in raw olive flounder meat, the system was tested using raw olive flounder with or without *K. septempunctata* infection. More than 5×10^5 *K. septempunctata* spores per gram of meat per assay was detected by the ELISA (FIG. 4). From 35 outbreaks of unidentified food-borne illness associated with the consumption of raw fish, it was observed that there were 2.4×10^6 (median) *Kudoa* spores in one gram of each food-borne outbreak sample (Kawai et al., 2012). The specificity of the CS#3 antiserum makes it appropriate for use in the estimation of *Kudoa* spore content in food-borne outbreak samples. In addition, the ELISA system established in this study might be applicable to the simultaneous evaluation of a large number of raw fish meat.

Competitive ELISA showed that CS#3 antiserum is less reactive with the spores of *K. iwatai*, which is a parasite of the *Perciformes* species (FIG. 2B). However, immunohistochemical staining revealed that CS#3 reacts with the spores of *K. lateolabralis* or *K. thyrsites*, which are parasites of the olive flounder (FIGS. 3C and 3D). Therefore, the antiserum appears to cross-react with at least two other species of *Kudoa* spores in raw fish meat. These results suggest that this anti-*Kudoa* serum recognizes a common structure of *Kudoa* that parasitizes raw fish meat. At present the toxicities of both *K. lateolabralis* and *K. thyrsites* are unknown. Nevertheless, this ELISA system seems to be useful for the monitoring and detection of *Kudoa* species in food-borne disease specimens. The preparation of rapid detection kits for *K. septempunctata* spores in *P. olivaceus* muscle tissue using immunochromatography is now in progress.

ACKNOWLEDGEMENTS

This work was supported in part by research projects of the Ministry of Health, Labour, and Grant-in-Aid for Scientific Research (KAKENHI) of the Ministry of Education, Culture, Sports, Science and Technology.

REFERENCES

Chase, J.C., Dawson-Coates, J.A., Haddow, J.D., Stewart, M.H., Haines, L.R., Whitaker, D.J., Ken, M.L., Olafson, R.W., and Pearson, T.W. (2001) Analysis of *Kudoa thyrsites* (Myxozoa: Myxosporea) spore antigens using monoclonal antibodies. *Dis. Aquat. Organ.*, **45**, 121-129.

Kawai, T., Sekizuka, T., Yahata, Y., Kuroda, M., Kumeda, Y., Iijima, Y., Kamata, Y., Sugita-Konishi, Y., and Ohnishi, T. (2012) Identification of *Kudoa septempunctata* as the causative agent of novel food poisoning outbreaks in Japan by consumption of Paralichthys olivaceus in raw fish. *Clin. Infect. Dis.*, **54**, 1046-1052.

Kikuchi, Y., Irie, M,, Yoshimatsu, K., Ishimaru, K., Shimomura, K., Satake, M., Sueyoshi, S., Tanno, M., Kamiya, S., Sawada, J., and Terao, T. (1991) A monoclonal antibody to scopolamine and its use for competitive enzyme-linked immunosorbent assay. *Phytochemistry*, **30**, 3273-3276.

Matsuda, H., Mitsuda, H., Nakamura, N., Furusawa, S., Mohri, S., and Kitamoto, T. (1999) A chicken monoclonal antibody with specificity for the N-terminal of human prion protein. *FEMS Immunol. Med. Microbiol.*, **23**, 189-194.

Matsukane, Y., Sato, H., Tanaka, S., Kamata, Y., and Sugita-Konishi, Y. (2010) *Kudoa septempunctata* n. sp. (Myxosporea: Multivalvulida) from an aquacultured olive flounder (*Paralichthys olivaceus*) imported from Korea. *Parasitol. Res.*, **107**, 865-872.

Ohnishi, T. (2012) Toxicity and detection method of Myxosporean parasite. *Jpn. J. Food Microbiol.*, **29**, 61-64.

Ohnishi, T., Kikuchi, Y., Furusawa, H., Kamata, Y., and Sugita-Konishi, Y. (2013) *Kudoa septempunctata* invasion increases the permeability of human intestinal epithelial monolayer. *Foodborne Pathogens Dis.*, **10**, 137-142.

Sumi, Y., Kawahara, S., Kikuchi, Y., Sawada, J., Suzuki, T., and Suzuki, K.T. (1993) Histochemical and immunohistochemical localization of copper, iron and metallothionein in the liver and kidney of LEC rats. *Acta Histochem. Cytochem.*, **26**, 5-9.

FOODBORNE PATHOGENS AND DISEASE
Volume 10, Number 2, 2013

DOI: 10.1089/fpd.2012.1294

Kudoa septempunctata Invasion Increases the Permeability of Human Intestinal Epithelial Monolayer

Takahiro Ohnishi, Yutaka Kikuchi, Hiroko Furusawa, Yoichi Kamata, and Yoshiko Sugita-Konishi

Abstract

Kudoa septempunctata is a myxosporean parasite of *Paralichthys olivaceus* (olive flounder) and causes a foodborne illness that affects more than 100 cases in Japan each year. We previously reported that the consumption of raw olive flounder meat containing a high concentration of *K. septempunctata* spores induces transient but severe diarrhea and emesis through an unknown mechanism. Here, we demonstrate that *K. septempunctata* sporoplasm plays an important role in mediating the toxicity of *K. septempunctata*. When *K. septempunctata* spores were inoculated in Caco-2 human intestinal cells, *K. septempunctata* sporoplasms were released from spores, and they invaded the cells. Electron microscopic observations revealed that the sporoplasm invasion severely damaged the Caco-2 cells. The inoculation of *K. septempunctata* spores eliminated the transepithelial electrical resistance (TER) across the cell monolayer. Inhibiting the invasion of the sporoplasms prevented the observed loss in cell layer integrity, as illustrated by the rapid elimination of the TER. These results suggest that the invasion by sporoplasms severely damaged individual intestinal cells, resulting in a loss of cell monolayer integrity.

Introduction

Outbreaks of an unidentified foodborne disease associated with the consumption of raw *Paralichthys olivaceus* (olive flounder) have increased in Japan and average more than 100 cases per year; however, no known agents of foodborne disease, such as foodborne bacteria, viruses, or toxic chemicals, have been recovered from the foods ingested by affected individuals (Kawai *et al.*, 2012). The lag phase is only 1–20 h, and symptoms include transient but severe diarrhea and emesis (Kawai *et al.*, 2012). The causative agent of this disease has been long debated, but in our previous study, we demonstrated that *Kudoa septempunctata*, a recently described myxosporean species, is the causative agent of this novel foodborne disease (Kawai *et al.*, 2012).

The *K. septempunctata* spore has six or seven shell valves and polar capsules (Matsukane *et al.*, 2010). The spore of this parasite has a pouch-like shape and is about 10 μm in diameter (Matsukane *et al.*, 2010). *K. septempunctata* cells dwell in the trunk muscles of *P. olivaceus*, and its spores are found uniformly distributed throughout *P. olivaceus* muscles. It has been suggested that the life cycle of *K. septempunctata* is maintained between *P. olivaceus* and an oligochaete or polychaete worm, but this has not been thoroughly studied (El Matbouli *et al.*, 1998). Oral administration of *K. septempunctata* spores to suckling mice caused fluid accumulation in the gut within 1.5 h of administration, with recovery after 4 h (Kawai *et al.*, 2012). The feeding of olive founder meat containing *K. septempunctata* spores to house musk shrews induced vomiting after 20–30 min with recovery within 2 h (Kawai *et al.*, 2012). Together, these experiments demonstrate the short lag phase and quick recovery from this parasite, and the symptoms observed in the animal experiments coincide with the symptoms exhibited by human patients with this disease (Kawai *et al.*, 2012). Despite this research, however, the mechanisms of this disease are poorly understood. Many species of protozoa induce diarrhea. In fact, protozoa such as *Giardia lamblia*, *Toxoplasma gondii*, *Cryptosporidium parvum*, and *Entamoeba histolytica* can invade and reproduce in the intestine, which is a cause of infection-associated diarrhea (Brandborg *et al.*, 1967; Chen *et al.*, 2000; Dubey *et al.*, 1997; Saha *et al.*, 1977; Yoshida *et al.*, 2011). Since some myxosporean parasites invade and reproduce in the intestinal cells of the worm (El Matbouli *et al.*, 1998), it has been hypothesized that *Kudoa* spp. are also able to invade the intestinal cell. However, it has not been shown whether *K. septempunctata* is able to invade and reproduce in the intestine of the worm. Moreover, we know that *K. septempunctata* causes disease in humans, but the behavior of *K. septempunctata* in the human intestine has not been well studied. In order to examine possible mechanisms by which *K. septempunctata* may cause diarrhea, we studied the toxicity and behavior of *K. septempunctata* in cultured human intestinal cells.

Division of Microbiology, National Institute of Health Sciences, Tokyo, Japan.

Materials and Methods

Cell monolayer permeability assay

The human adenocarcinoma cell line Caco-2 cells (American Type Cell Culture [ATCC] HTB37) was normally maintained in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; Life Technologies) and 1% non-essential amino acids (Life Technologies) at 37°C in 5% CO_2. For differentiation, Caco-2 cells were suspended in enterocyte differentiation medium (EDM; BD Biosciences, Bedford, MA) supplemented with 0.08% MITO+ Serum Extender (BD Biosciences) and antibiotics, and seeded at 2×10^5 cells/well in Biocoat cell culture inserts (pore size of 1 μm; BD Biosciences). Cultures were incubated at 37°C for 3 days, and the culture medium was replaced every day. After 3 days, the transepithelial electrical resistance (TER) values were measured using Millicell-ERS (Milipore, Billerica, MA) to ensure the state of differentiation and cell monolayer integrity. Inserts that showed a TER of >800 Ω cm^2 were subjected to experimentation. *P. olivaceus* infected with *K. septempunctata* were purchased from a local fish market in Japan, and *K. septempunctata* spores were purified as described previously (Kawai *et al.*, 2012). Purified *K. septempunctata* spores (5×10^5 spores/well) were inoculated in cell culture inserts, and the TER was measured every hour.

In some experiments, purified *K. septempunctata* spores were suspended in phosphate-buffered saline (PBS) and sonicated by Branson Sonifier 200 (Branson Ultrasonics, Danbury, CT). The sonicated spores (equivalent to 5×10^5 spores/well) were inoculated in cell culture inserts, and the TER was measured.

To examine toxin production, *K. septempunctata* spores (1×10^7 spores/mL) were suspended in EDM supplemented with 0.08% MITO+ Serum Extender. The suspension was incubated 37°C for 18 h. After centrifugation at 1,500×*g* for 15 min at 4°C, the supernatant was recovered. The culture medium of differentiated Caco-2 cells was replaced with the supernatant of *K. septempunctata* spores, and the TER was measured.

Sporoplasm release assay

K. septempunctata spores purified as above were suspended in PBS, H_2O, or H_2O (pH 2) to a final concentration of 10^7 spores/mL, with or without protease inhibitor (Nacalai Tesque, Inc., Kyoto, Japan). These suspensions were then incubated with FCS, trypsin (Thermo Fisher Scientific Inc., Rockford, IL), or pepsin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at the final concentration indicated in Table 1. The suspensions were incubated at room temperature for 1 h, and sporoplasm release was observed under a bright-field microscope. The release of sporoplasm was counted in 10 randomly selected microscopic fields, and a positive result was indicated by sporoplasm release from >30% of the spores.

Transmission electron microscopy (TEM)

After plating Caco-2 cells (8×10^5 cells/dish) in 6-cm cell culture dish coated with Cellmatrix type I collagen solution (Nitta Gelatin Inc., Osaka, Japan), the cells were differentiated as described above. Three days later, the purified *K. septempunctata* spores (6×10^6 spores/dish) were inoculated into cells, and the cultures were incubated at 37°C for 1 h. After incubation, the cultures were fixed with 2% paraformaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer (PB) pH 7.4 at 37°C, and incubated at 4°C for 30 min to lower the temperature. Thereafter, they were fixed with 2% glutaraldehyde in 0.1 M PB at 4°C overnight. Following fixation, the samples were rinsed three times with 0.1 M PB for 30 min each, followed by post fixation with 2% osmium tetroxide in 0.1 M PB at 4°C for 1 h. The samples were dehydrated through an ethanol series (50%, 70%, 90%, 100%). The schedule was as follows: 50% and 70% for 15 min each at 4°C, 90% for 5 min at room temperature, and three changes of 100% for 5 min each at room temperature. After dehydration, the samples were transferred to a resin (Quetol-812; Nisshin EM Co., Tokyo, Japan) and polymerized at 60°C for 48 h. Ultra-thin sections (70 nm) of the blocks were prepared with a diamond knife by using an ultramicrotome (Ultracut UCT; Leica Microsystems GmbH, Wetzlar, Germany), and the sections were placed on copper grids, stained with 2% uranyl acetate at room temperature for 15 min, and then rinsed with distilled water, followed by secondary staining with lead stain solution (Sigma-Aldrich Co., St. Louis, MO) at room temperature for 3 min. The grids were observed by TEM (JEM-1200EX; JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV. Digital images were taken with a charge-coupled device (CCD) camera (Veleta; Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Table 1. Stimulant for Sporoplasm Release

Stimulants	*Release of sporoplasm*
10% FCS in PBS	+
10% FCS+Protease inhibitor cocktail in PBS	–
Trypsin (400 μg/mL) in PBS	+
PBS	–
Pepsin (5 μg/mL) in H_2O	–
Pepsin (5 μg/mL) in H_2O, pH 2	–
H_2O	–

FCS, fetal calf serum; PBS, phosphate-buffered saline.

Scanning electron microscopy (SEM)

Caco-2 cells (8×10^5 cells/dish) were seeded and differentiated as mentioned above. Three days later, the purified *K. septempunctata* spores (6×10^6 spores/dish) were inoculated into cells, and the cultures were incubated at 37°C for 1 h. After incubation, the samples were fixed and dehydrated as described above. The samples were treated with tert-butyl alcohol three times for 30 min each, followed by freezing at 4°C. The frozen samples were dried using a dry vacuum pump (DAP-6D; ULVAC KIKO, Inc., Miyazaki, Japan) with slow decompression. After drying, the samples were coated with a thin layer (30 nm) of osmium by using an osmium plasma coater (NL-OPC80NS; Nippon Laser & Electronic Laboratory, Nagoya, Japan). The samples were observed by SEM (S-800; Hitachi High-Tech Fielding Co., Tokyo, Japan) at an acceleration voltage of 10 kV.

Inhibition of sporoplasm release and confocal microscopy

After plating Caco-2 cells (4×10^5 cells/culture) in a cover glass coated with Cellmatrix type I collagen solution, the cells were differentiated as described above. Three days later, the purified *K. septempunctata* spores (3×10^6 spores/culture) were inoculated into Caco-2 cells with or without cytochalasin D (Wako Pure Chemicals) dissolved in DMSO (Wako Pure Chemicals), and the cultures were incubated at 37°C for 1 or 2 h. The cells were washed two times with PBS and fixed with 4% paraformaldehyde in PB for 30 min at room temperature. After fixation, the cells were washed two times with PBS, and then incubated with chicken anti–*K. septempunctata* antiserum in PBS containing 10% normal goat serum at 4°C for 18 h. Chicken anti–*Kudoa* antiserum was raised against sonicated *K. septempunctata* spores according to the method described by Matsuda *et al.* (1999). After incubation, the cells were washed five times with PBS, and then incubated with Alexa 488 goat anti–chicken IgG (Life Technologies) and rhodamine phalloidin (Life Technologies) in PBS containing 10% normal goat serum at 4°C for 1 h in the dark. After five washes with PBS, the cover slips were mounted on slide glass with SlowFade Gold antifade reagent (Life Technologies). Confocal microscopy and differential interference contrast microscopy were conducted with a confocal laser scanning microscope (FV1000-D; Olympus Co., Tokyo, Japan).

To examine the effects of cytochalasin D, we differentiated Caco-2 in Biocoat cell culture inserts as mentioned above, inoculated the spores of *K. septempunctata* to Caco-2 cells in the presence of cytochalasin D at the concentration indicated, and measured the TER. Since cytochalasin D and DMSO showed weak toxicity on Caco-2 cells, TER was presented as a ratio to the culture without inoculation of spores.

Results and Discussion

Effects of K. septempunctata *on the permeability across the Caco-2 cell monolayer*

Caco-2 cell line has been widely used as an *in vitro* model for evaluating intestinal permeability because of its structural and functional similarity to mature intestinal epithelium (Lytton *et al.*, 2005; Dickman *et al.*, 2000). The cell monolayer permeability assay was performed to investigate the toxicity of *K. septempunctata* towards Caco-2 cells. We obtained *P. olivaceus* infected with *K. septempunctata* and purified spores from the trunk muscles. The spores were inoculated in Caco-2 cells, and the TER across the Caco-2 cell monolayer was measured as an indicator of permeability. The inoculation of purified *K. septempunctata* spores rapidly decreased TER by 80% within 1 h, indicating an increase in the permeability of the Caco-2 cell monolayer (Fig. 1). Our previous study showed that freezing inactivates *K. septempunctata* (Kawai *et al.*, 2012); indeed, *K. septempunctata* purified from frozen *P. olivaceus* did not affect TER (Fig. 1). To determine whether *K. septempunctata* produces a toxic substance, spores were incubated in EDM at 37°C for 18 h, and the supernatant was recovered. When the culture medium from the Caco-2 cells was replaced with the supernatant from the spore, TER was not changed (Fig. 1). The inoculation of sonicated *K. septempunctata* spores also did not affect TER. These results suggested that *K. septempunctata* reduced TER by mechanisms other than production of toxin.

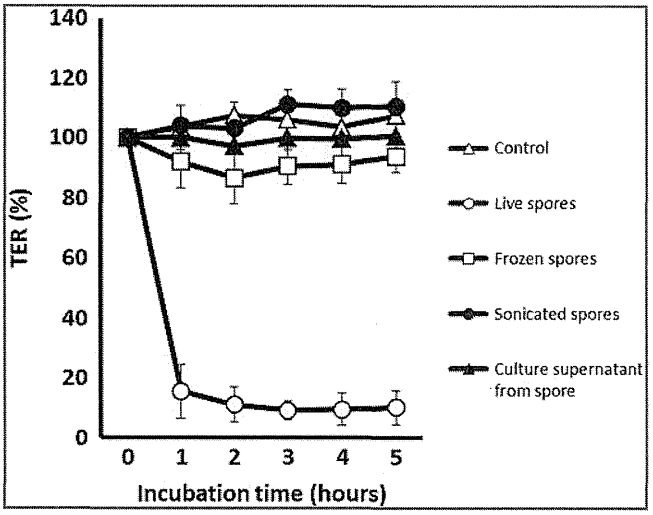

FIG. 1. Increased permeability across the Caco-2 cell monolayer induced by *Kudoa septempunctata* spores. Differentiated Caco-2 cells were treated with purified live spores, frozen spores, sonicated spores, or culture supernatant from spores. Transepithelial electrical resistance (TER) was measured for up to 5 h. TER at time 0 is represented as 100%. Values are the mean ± SD from three independent experiments.

Release of sporoplasms from spores

To further investigate whether *K. septempunctata* spores directly affect Caco-2 cells, we inoculated Caco-2 cells with spores and observed the cells under the microscope (Fig. 2). Within 1 h after inoculation, we observed the release of protoplasm from the spores on the surface of Caco-2 cells (Fig. 2, left panel), and this protoplasm, as well as the spores themselves, reacted with anti–*Kudoa* antiserum (Fig. 2, right panel). As it has been reported previously that some myxosporean parasites release sporoplasm into the host intestine (El Matbouli *et al.*, 1998), we believe that this material is likely the sporoplasms of *K. septempunctata.*

Next, we aimed to determine the stimulant responsible for the release of sporoplasms. Since the release of sporoplasm has been observed on Caco-2 cells cultured in the presence of FCS (T. Ohnishi, unpublished data), we first treated *K. septempunctata* spores with 10% FCS in PBS (Table 1). This treatment induced the release of sporoplasm, and the release was inhibited by the addition of a protease inhibitor cocktail (Table 1). These results indicated that one of the inducers is a protease present in FCS. We were not able to identify the specific protease in FCS, but a protease inhibitor cocktail blocking serine-, cysteine-, and trypsin-like protease activities inhibited sporoplasm release in FCS. Since one of the major proteases secreted in the intestine is trypsin, we treated *K. septempunctata* spores with trypsin and found that trypsin also stimulated sporoplasm release (Table 1). Interestingly, treatment with the protease pepsin did not induce the release of sporoplasm. These results suggest that some proteases secreted in the intestine can induce the release of sporoplasms from the spore. In our Caco-2 cell model, the cells were

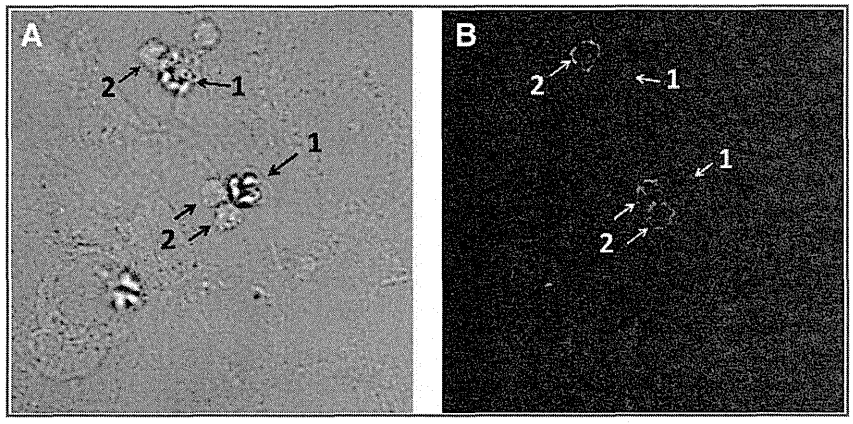

FIG. 2. Release of sporoplasms from spores induced by inoculation of spores into Caco-2 cells. Differentiated Caco-2 cells were inoculated with *Kudoa septempunctata* spores and incubated for 1 h at 37°C. Samples were stained with α–*K. septempunctata* antiserum and rhodamine phalloidin, and analyzed by differential interference contrast microscopy **(A)** and confocal microscopy **(B).** Since the depth of field of confocal laser scanning microscope is very small, the staining of actin hardly appears in panel B. 1, spore; 2, sporoplasm.

differentiated under serum-free conditions prior to experiments. However, it has been shown that the active forms of glycosylphosphatidylinisotol (GPI)–anchored trypsin-like proteases are expressed on the apical surface of Caco-2 cells (Friis *et al.*, 2011). Caco-2 cells also express other proteases on their apical surface (Buzza *et al.*, 2010; Wang *et al.*, 2009). Therefore, we hypothesize that *K. septempunctata* spores utilize the proteases expressed on the apical surface of the intestinal cells to induce the release of sporoplasms in our Caco-2 cell model.

We used electron microscopy to investigate the role of sporoplasms in the pathogenesis (Fig. 3). The electron microscopy showed that *K. septempunctata* spores have an asymmetrically stellate shape in apical view (Fig. 3A) (Matsukane *et al.*, 2010). When *K. septempunctata* spores were inoculated in Caco-2 cells, TEM confirmed the release of

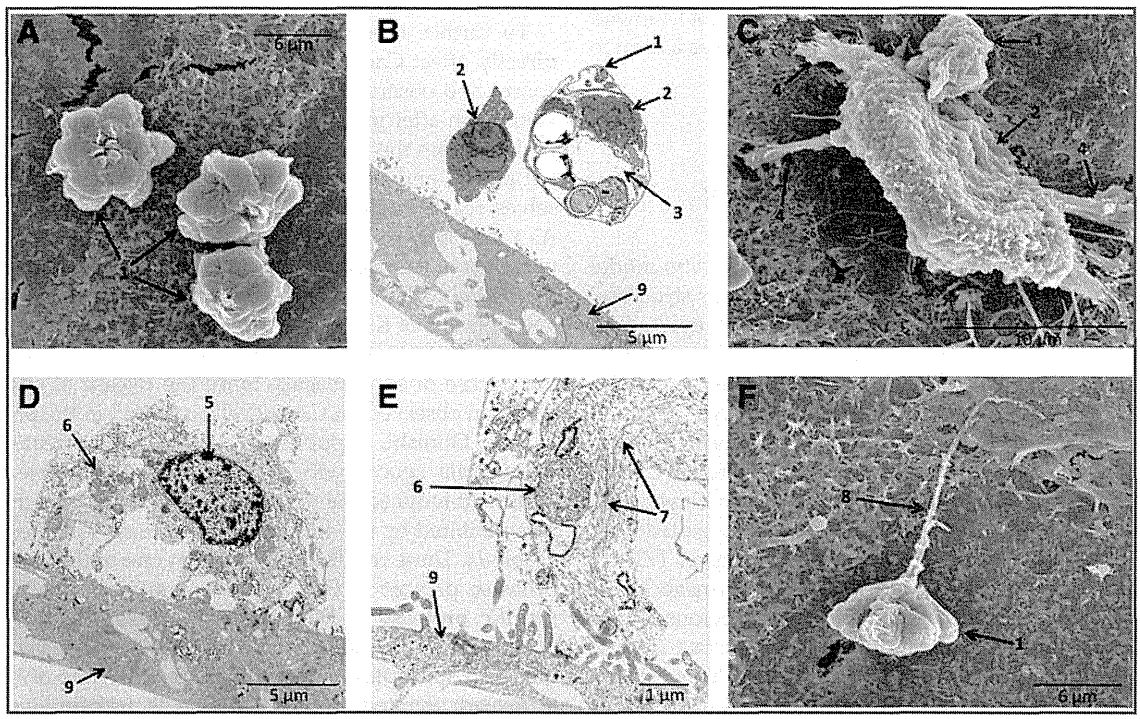

FIG. 3. Morphological observations of *Kudoa septempunctata* by electron microscopy. Differentiated Caco-2 cells were inoculated with *K. septempunctata* spores and incubated for 1 h at 37°C. Samples were observed using both scanning electron microscopy (SEM) and transmission electron microscopy (TEM). **(A)** SEM of *K. septempunctata* spores. **(B)** TEM of *K. septempunctata* spore and sporoplasm. **(C)** SEM of *K. septempunctata* spore and sporoplasm. **(D)** TEM of sporoplasm. **(E)** Magnification of panel D. **(F)** SEM of spore. 1, spore; 2, sporoplasm; 3, inner space for sporoplasm; 4, pseudopod; 5, cell nucleus; 6, mitochondria like organelle; 7, cytoskeleton; 8, polar filament; 9, Caco-2 cell.

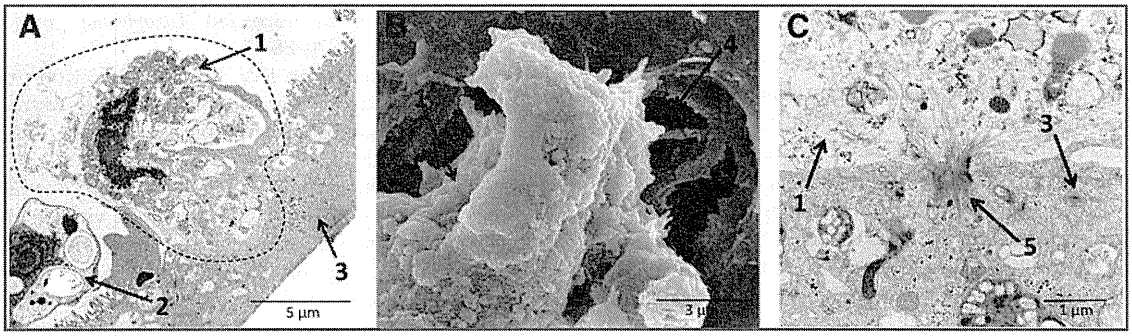

FIG. 4. Cell damage caused by sporoplasm invasion of Caco-2 cells. Differentiated Caco-2 cells were inoculated with *Kudoa septempunctata* spores and incubated for 1 h at 37°C. Samples were observed using both scanning electron microscopy (SEM) and transmission electron microscopy (TEM). **(A)** TEM of *K. septempunctata* sporoplasm. **(B)** SEM of *K. septempunctata* sporoplasm. **(C)** TEM of *K. septempunctata* sporoplasm. 1, sporoplasm; 2, spore; 3, Caco-2 cell; 4, hole on Caco-2 cell; 5, point of invasion. Dotted line indicates the sporoplasm outline.

sporoplasms on the Caco-2 cell monolayer (Fig. 3B). Cross section analysis of the spore revealed two spaces for the sporoplasms (Fig. 3B). After release from the spore, the sporoplasm swelled and obtained an amoeba-like shape (Fig. 3C,D). Dense cytoskeletons were developed in the sporoplasm (Fig. 3E), and the sporoplasm developed pseudopodia (Fig. 3C). The discharge of polar filaments from the spore was also observed, and the polar filaments adhered to the cell surface (Fig. 3F).

Sporoplasm invasion of Caco-2 cells

During infection, examination of cross section using TEM indicated that the sporoplasm intruded directly into the cytoplasm of Caco-2 cells (Fig. 4A). Examination of infecting sporoplasms by SEM suggested that this intrusion resulted in large holes on the Caco-2 cell surface (Fig. 4B). Following release from the spore, we observed that the cytoskeleton within sporoplasms became more defined (Fig. 4C). This reflects the condensation of actin filaments (Alama-Bermejo *et al.*, 2012), likely to aid in the movement of the sporoplasm in the Caco-2 cell. We hypothesized that inhibiting actin polymerization in the sporoplasm would prevent the movement of sporoplasm, resulting in the inability to release from spores or invade cells. When we inoculated *K. septempunctata* in Caco-2 cells in the presence of cytochalasin D, treatment with 2 µM cytochalasin D completely inhibited the release of sporoplasm from spores (Fig. 5A, lower panel). In contrast, 1 h after infection without cytochalasin D, the sporoplasms had reached the basolateral side of the Caco-2 cells, as evidenced by confocal microscopy (Fig. 5A, upper panel). The reduction of TER induced by *K. septempunctata* was suppressed by cytochalasin D in a dose-dependent manner and was completely inhibited at a concentration of 2 µM (Fig. 5B). Collectively, our results demonstrate that invasion by *K. septempunctata* sporoplasms eliminated TER across the Caco-2 cell monolayer. Stopping cell invasion either by inactivating the spores or by inhibiting the release of sporoplasm prevented loss in TER. The sporoplasm of *K. septempunctata* very quickly intruded into Caco-2 cells and reached the basolateral side of Caco-2 cells within 1 h of infection. This rapid invasion coincides with the quick reduction of TER and the short lag phase before the appearance of clinical symptoms.

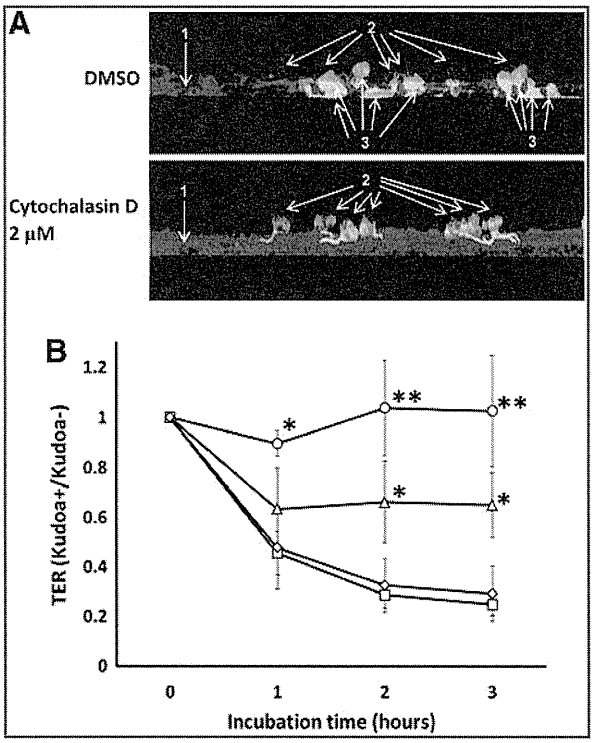

FIG. 5. Effect of increasing concentrations of cytochalasin D on sporoplasm-mediated reduction in the transepithelial electrical resistance. **(A)** *Kudoa septempunctata* spores were inoculated onto differentiated Caco-2 monolayers in medium containing dimethyl sulfoxide (DMSO) and/or cytochalasin D and incubated for 1 h at 37°C. Inoculated monolayers were stained with α–*K. septempunctata* antiserum and rhodamine phalloidin, and analyzed by confocal microscopy. 1, cross section of Caco-2 cell monolayer (actin); 2, spore; 3, sporoplasm. **(B)** *K. septempunctata* spores were inoculated in differentiated Caco-2 cells with various concentrations of cytochalasin D (2 µM, ○), (0.2 µM, △), (0.02 µM, □), or DMSO (◇). Transepithelial electrical resistance (TER) was measured for up to 3 h. Because both cytochalasin D and DMSO affected permeability of Caco-2 cells, TER is presented as a ratio to the culture without the inoculation of spores. Values are the mean ± SD from three independent experiments. $*p < 0.05$; $**p < 0.01$ versus DMSO (Student's *t*-test).

Conclusion

Our results demonstrate that the invasion of human epithelial cells by *K. septempunctata* sporoplasms occurs quite rapidly, resulting in the termination of monolayer confluence as illustrated by the rapid loss of the TER due to severe damage of individual cells within the monolayer. Rapid invasion of gut epithelial cells by *K. septempunctata* sporoplasms may be a contributing factor to the diarrhea associated with this pathogen, although additional work will be required to further elucidate the mechanisms of pathogenesis in *K. septempunctata*.

Acknowledgments

We thank Yutaka Fukuda, Oita Prefectural Agriculture, Forestry and Fisheries Research, and Mitsuru Ototake and Hiroshi Sako, National Research Institute of Aquaculture, for valuable advices. This research was supported by the Ministry of Health, Labour and Welfare, Japan (Health Labour Sciences Research Grant H23-shyokuhin-ippan-007).

Disclosure Statement

No competing financial interests exist.

References

Alama-Bermejo G, Bron JE, Raga JA, Holzer AS. 3D Morphology, ultrastructure and development of *Ceratomyxa puntazzi* stages: First insights into the mechanisms of motility and budding in the Myxozoa. PLoS One 2012;7:e32679.

Brandborg LL, Tankersley CB, Gottieb S, Barancik M, Sartor VE. Histological demonstration of mucosal invasion by *Giardia lamblia* in man. Gastroenterology 1967;52:143–150.

Buzza MS, Netzel-Arnett S, Shea-Donohue T, Zhao A, Lin CY, List K, Szabo R, Fasano A, Bugge TH, Antalis TM. Membrane-anchored serine protease matriptase regulates epithelial barrier formation and permeability in the intestine. Proc Natl Acad Sci USA 2010;107:4200–4205.

Chen XM, LaRusso NF. Mechanisms of attachment and internalization of *Cryptosporidium parvum* to biliary and intestinal epithelial cells. Gastroenterology 2000;118:368–379.

Dickman KG, Hempson SJ, Anderson J, Lippe S, Zhao L, Burakoff R, Shaw RD. Rotavirus alters paracellular permeability and energy metabolism in Caco-2 cells. Am J Physiol Gastrointest Liver Physiol 2000;279:G757–G766.

Dubey JP, Speer CA, Shen SK, Kwok OC, Blixt JA. Oocyst-induced murine toxoplasmosis: Life cycle, pathogenicity, and stage conversion in mice fed *Toxoplasma gondii* oocysts. J Parasitol 1997;83:870–882.

El Matbouli M, Hoffmann RW. Light and electron microscopic studies on the chronological development of *Myxobolus cerebralis* to the actinosporean stage in Tubifex tubifex. Int J Parasitol 1998;28:195–217.

Friis S, Godiksen S, Bornholdt J, Selzer-Plon J, Rasmussen HB, Bugge TH, Lin CY, Vogel LK. Transport via the transcytotic pathway makes prostasin available as a substrate for matriptase. J Biol Chem 2011;286:5793–5802.

Kawai T, Sekizuka T, Yahata Y, Kuroda M, Kumeda Y, Iijima Y, Kamata Y, Sugita-Konishi Y, Ohnishi T. Identification of *Kudoa septempunctata* as the causative agent of novel food poisoning outbreaks in Japan by consumption of *Paralichthys olivaceus* in raw fish. Clin Infect Dis 2012;54:1046–1052.

Lytton SD, Fischer W, Nagel W, Haas R, Beck FX. Production of ammonium by *Helicobacter pylori* mediates occludin processing and disruption of tight junctions in Caco-2 cells. Microbiology 2005;151:3267–3276.

Matsuda H, Mitsuda H, Nakamura N, Furusawa S, Mohri S, Kitamoto T. A chicken monoclonal antibody with specificity for the N-terminal of human prion protein. FEMS Immunol Med Microbiol 1999;23:189–194.

Matsukane Y, Sato H, Tanaka S, Kamata Y, Sugita-Konishi Y. *Kudoa septempunctata* n. sp. (Myxosporea: Multivalvulida) from an aquacultured olive flounder (*Paralichthys olivaceus*) imported from Korea. Parasitol Res 2010;107:865–872.

Saha TK, Ghosh TK. Invasion of small intestinal mucosa by *Giardia lamblia* in man. Gastroenterology 1977;72:402–405.

Wang JK, Lee MS, Tseng IC, Chou FP, Chen YW, Fulton A, Lee HS, Chen CJ, Johnson MD, Lin CY. Polarized epithelial cells secrete matriptase as a consequence of zymogen activation and HAI-1-mediated inhibition. Am J Physiol Cell Physiol 2009;297:C459–C470.

Yoshida N, Tyler KM, Llewellyn MS. Invasion mechanisms among emerging food-borne protozoan parasites. Trends Parasitol 2011;27:459–466.

Address correspondence to:
Takahiro Ohnishi, PhD
Division of Microbiology
National Institute of Health Sciences
1-18-1 Kamiyoga
Setagaya, Tokyo 158-8501, Japan

E-mail: ohnisi@nihs.go.jp