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Production of a novel monoclonal antibody applicable for an immunochromatographic assay for *Kudoa septempunctata* spores contaminating the raw olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

Kudoa septempunctata, a myxosporean parasite of the olive flounder (*Paralichthys olivaceus*), causes foodborne gastroenteritis after ingestion of contaminated raw flounder. Available methods to detect *K. septempunctata* require expensive equipment, well-trained personnel, and lengthy procedures. Here we generated a novel monoclonal antibody (MAb 15G11) against *K. septempunctata* and used it to produce a prototype immunochromatographic assay (prototype Kudoa-ICA). Within 15 min, the prototype Kudoa-ICA detected $\geq 1.0 \times 10^5$ spores/mL in a spore suspension and $\geq 2.0 \times 10^4$ spores/g of *P. olivaceus* muscle. The prototype Kudoa-ICA weakly cross-reacted with spores of *K. lateolabracis* and *K. iwatai*. cDNA sequence, expression, and western blot analyses revealed that MAb 15G11 detected an approximately 24-kDa protein encoded by a 573 bp mRNA. The cDNA nucleotide and predicted amino acid sequences were not significantly similar to any sequence in the GeneBank database. Immunoelectron microscopy revealed that MAb 15G11 reacted with the sporoplasmic cells and mainly with the capsulogenic cells of the *K. septempunctata* spore. Although the Kudoa-ICA was weakly cross-reactive with two other *Kudoa* species, it detected $> 1.0 \times 10^6$ spores/g of *K. septempunctata* in *P. olivaceus* muscle, which is the criterion used to indicate a violation of the Food Hygiene Law of Japan. We conclude that MAb 15G11 may be suitable for use in an immunochromatographic assay for screening *P. olivaceus* muscle contaminated with *K. septempunctata* at food distribution sites such as food wholesalers, grocery stores, and restaurants.

1. Introduction

Kudoa septempunctata is a myxosporean parasite, which infects the muscle of the olive flounder (*Paralichthys olivaceus*) and causes foodborne gastroenteritis (Kawai et al., 2012; Matsukane et al., 2010). Similarly, *K. thyrsites* and *K. lateolabracis* infect the muscle of *P. olivaceus*, whereas *K. yasunagai* infects the brain (Grabner et al., 2012; Sugita-Konishi et al., 2014; Whipps et al., 2004; Yokoyama et al., 2004). Among them, only *K. septempunctata* causes foodborne disease. Individuals who ingest raw *P. olivaceus* muscle containing *K. septempunctata* spores experience acute diarrhea and vomiting within 2–20 h (Kawai et al., 2012; Sugita-Konishi et al., 2014).

A study using the suckling-mouse test demonstrated dose-dependent diarrheagenic activity of *K. septempunctata* spores (Kawai et al., 2012). An analysis of 35 outbreaks of foodborne disease associated with the consumption of raw *P. olivaceus* muscle detected a median concentration of 2.4×10^6 spores/g of *K. septempunctata* (Kawai et al., 2012). Epidemiological analysis of 59 cases collected by a local public health

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center in Japan estimated that the ingestion threshold for developing symptoms is 7.2×10^7 *K. septempunctata* spores per person (Yahata et al., 2015). Although Chung and Bae (2017) reviewed two articles that reported no significant pathogenicity of *K. septempunctata* (Chung and Bae, 2017), the suckling-mouse test and epidemiological analysis provide evidence for an association between the number of *K. septempunctata* spores ingested by a patient and the onset of clinical symptoms (Kawai et al., 2012; Yahata et al., 2015). The Ministry of Health, Labor, and Welfare of Japan reported that 33, 41, 21, 43, 17, and 22 outbreaks of foodborne disease were caused by *K. septempunctata* in 2011, 2012, 2013, 2014, 2015, and 2016, respectively, involving 473, 418, 244, 429, 169, and 259 individuals, respectively. To prevent outbreaks of foodborne disease, it is a violation of the Food Hygiene Law of Japan in 2012 (Ohnishi et al., 2016) to sell *P. olivaceus* contaminated with $> 1.0 \times 10^6$ spores/g muscle.

Although heating at 95 °C for 10 min or freezing at − 80 °C overnight inactivates *K. septempunctata* spores (Sugita-Konishi et al., 2014), these treatments spoil the texture of raw *P. olivaceus* muscle. Moreover, in Japan, *P. olivaceus* muscle is usually consumed raw such as in sushi (cooked rice with sliced raw fish) and sashimi (sliced raw fish), and heating and freezing of *P. olivaceus* muscle are not widely practiced. Therefore, preventing or limiting outbreaks of foodborne disease caused by *K. septempunctata* requires a rapid and easy method to safely evaluate *P. olivaceus* muscle at food distribution sites such as wholesalers, grocery stores, and restaurants.

The aquaculture industry requires rapid and easy detection methods. To screen for *P. olivaceus* contaminated with *K. septempunctata*, the method adopted by the Ministry of Agriculture, Forestry, and Fisheries of Japan employs optical microscopic analysis of smears of specimens collected from an incision site near the medulla oblongata. However, this method may contaminate samples with *K. yasunagai*, which can infect the brain of *P. olivaceus*. The spores of *K. septempunctata* and *K. yasunagai* comprise six or seven and six to eight shell valves, respectively, each with an equal number of polar capsules (Grabner et al., 2012; Kasai et al., 2016; Matsukane et al., 2010; Sugita-Konishi et al., 2014; Whipps et al., 2004). It is therefore difficult to distinguish the spores of *K. yasunagai* from those of *K. septempunctata* using optical microscopic analysis.

Highly sensitive and specific methods that detect DNA, including conventional PCR (Grabner et al., 2012) and real-time PCR (Harada et al., 2012), nucleic acid sequence-based amplification-nucleic acid chromatography (NASBA-NAC) (Sugita-Konishi et al., 2015), and loop-mediated isothermal amplification (LAMP) (Jeon et al., 2014) are used to diagnose foodborne disease caused by *K. septempunctata*. However, the use of these methods at the sites of food distribution is difficult, because they require expensive equipment, well-trained people, and time-consuming procedures. To address this problem, an immunochromatographic assay (ICA) was developed to detect pathogens (Posthuma-Trumpie et al., 2009). The benefits of the ICA include a simple one-step procedure, rapid test time of approximately 15 min, and easy acquisition of results by visual observation of an immnunoreactive band on the ICA strip (Yonekita et al., 2013). These attributes make the ICA suitable for screening *K. septempunctata* that contaminates *P. olivaceus* muscle at the sites of food distribution.

Here we generated a novel monoclonal antibody (MAb) against *K. septempunctata* spores, which was used to produce and evaluate a prototype immunochromatographic assay (prototype Kudoa-ICA) to detect *K. septempunctata*. We determined the cDNA sequence of the antigen recognized by the MAb and used immunoelectron microscopy (IEM) to detect the antigen in *K. septempunctata* spores.

2. Materials and methods

2.1. Parasite and fish samples

We used the spores of *K. septempunctata* and other *Kudoa* spp. including *K. thyrsites*, *K. lateolabracis*, *K. hexapunctata*, *K. yasunagai*, *K. iwatai*, and *K. amamiensis*. The spores of *K. septempunctata* and *K. thyrsites* were extracted from *P. olivaceus* muscle, *K. amamiensis* from amberjack (*Seriola quinqueradiata*) muscle, and *K. hexapunctata* from bluefin tuna (*Thunnus orientalis*) muscle, and these *Kudoa* spp. spores were purified using Percoll density gradient centrifugation as previously described (Kawai et al., 2012). The spores of *K. lateolabracis* were extracted from *P. olivaceus* muscle as previously described (Kawai et al., 2012) without Percoll purification. The spores of *K. yasunagai* and *K. iwatai* were collected by incision of each cyst harvested from the barbel eel (*Plotosus japonicus*) or *P. olivaceus* brain and sea breams (*Pagrus major* and *Acanthopagrus latus*) and sea bass (*Lateolabrax japonicus*) muscle, respectively. The spores were suspended in phosphate-buffered saline (PBS) and stored at − 20 °C. The spores used for the secondary selection of hybridomas were stored at 4 °C or − 20 °C.

K. septempunctata-contaminated *P. olivaceus* muscle, which were stored at 4 °C for 5 days after killing the fish, were used to evaluate the Kudao-ICA. The number of *Kudoa* spores was counted as previously described (Kawai et al., 2012).

The identification of *K. septempunctata* spores and determination of contamination of *P. olivaceus* muscle with *K. septempunctata* were performed using a real-time PCR assay described previously (Harada et al., 2012).

2.2. Preparation of MAbs against K. septempunctata spores

MAbs against *K. septempunctata* spores were prepared as previously described (Kawatsu et al., 2006) with modifications as follows: Female BALB/c mice (aged 8 weeks) were immunized intraperitoneally with 1.0×10^6 *K. septempunctata* spores emulsified with TiterMax Gold (TiterMAX USA Inc., Norcross, GA, USA). After 3, 5, 7, and 12 weeks, the mice were boosted intraperitoneally with the emulsified immunogen. At 6 weeks, the mice were bled, and the antibody titer of each serum sample was determined using an enzyme-linked immunosorbent assay (ELISA) performed as previously described (Sakata et al., 2012). A sonicated suspension of *K. septempunctata* spores was used to coat the wells of the ELISA plates. The mouse with the highest serum titer after 15 weeks was injected intraperitoneally with 2.0×10^6 spores of *K. septempunctata*.

Three days after this final immunization, spleen cells were fused with P3-X63-Ag8.U1 myeloma cells as previously described (Kawatsu et al., 2006). After fusion and cloning, culture supernatants were screened using the ELISA described above. To select hybridomas secreting antibodies reacting with various types of *K. septempunctata* spores but not with *P. olivaceus* muscle (described below), hybridoma culture supernatants were analyzed using a previously described ELISA (Sakata et al., 2012). Absorbance values ≥ 1.0 were defined as a positive reaction. In this ELISA, the wells of the plates were coated with homogenized *P. olivaceus* muscle devoid of *Kudoa* spp. spores, as confirmed using PCR (Grabner et al., 2012), and microscopic observations (Kawai et al., 2012). Alternatively, the wells were coated with preparations of *K. septempunctata* spores as follows: (a) untreated *K. septempunctata* spore suspension stored at − 20 °C or (b) 4 °C; (c) supernatant or (d) precipitate of a sonicated *K. septempunctata* spore suspension; (e) supernatant of a *K. septempunctata* spore suspension homogenized using a BioMasher II (Nippi Inc., Tokyo, Japan); (f) supernatant or (g) precipitate of vortexed *K. septempunctata* spores treated with 0.1% Triton X-100; (h) supernatant or (i) precipitate of vortexed *K. septempunctata* spores treated with 0.5% sodium cholate; and (j) supernatant of a boiled *K. septempunctata* spore suspension. Each supernatant and precipitate was prepared by centrifuging the samples at 15,000 × *g* for 5 min. The precipitate was suspended in PBS. After producing, purifying, and isotyping the MAbs as previously described (Kawatsu et al., 2006), the MAbs were conjugated to horseradish peroxidase (1 mg/mL) using a Peroxidase Labeling Kit-SH (Dojindo Molecular Technologies Inc., Kumamoto, Japan) according to the