Seroprevalences Against *Paracoccidioides cetii*: A Causative Agent for Paracoccidiomycosis Ceti (PCM-C) and *Coccidioides posadasii*; for Coccidioidomycosis (CCM) in Dall’s Porpoise (*Phocoenoides dalli*) and Harbor Porpoise (*Phocoena phocoena*) Stranded at Hokkaido, Japan

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Abstract  Paracoccidioidomycosis ceti (PCM-C) is a zoonotic mycosis characterized by chronic granulomatous cutaneous lesions in cetaceans. It is distributed worldwide and is caused by an unculturable fungus; *Paracoccidioides cetii*. On the other hand, coccidioidomycosis (CCM), caused by *Coccidioides* spp., is also a zoonotic and highly pathogenic fungal infection endemic in both American continents. Even though the Far East is not an endemic area of CCM, an autochthonous case has been reported in China. Although the seroprevalence against *P. cetii* in captive dolphins was 61.0%, there is no information on wild dolphins living in cold waters. The present study aimed to investigate the seroprevalence against *P. cetii* and *C. posadasii* in 15 Dall’s porpoises (*Phocoenoides dalli*) and 11 harbor porpoises (*Phocoena phocoena*) stranded in Hokkaido, Japan. The seroprevalence against *P. cetii* in the above dolphins was 26.9% (7/26), which was recorded only in Dall’s porpoises (7/15), and that against *C. posadasii* was 15.4% (4/26), three in Dall’s porpoises and one in harbor porpoise. The present study demonstrated positive seroprevalence against *P. cetii* and *C. posadasii* in wild cetaceans living in the subarctic areas of the Far East as the first records, and would issue the warning those who live in the area were exposed to the causative agent of CCM from seawater.
Keywords  Coccidiomycosis · Paracoccidioidomycosis ceti · Porpoise · Seroprevalence

Introduction

Paracoccidioidomycosis ceti (PCM-C) is a zoonotic mycosis characterized by chronic granulomatous keloidal dermatitis in cetaceans that has been reported worldwide [1], and is treated as a zoonosis, based on a human case of a dolphin trainer who handled an infected animal [2].

In 2018, the fungal name of the causative agent of PCM-C was given as Paracoccidioides brasiliensis var. ceti, which has been the popular name up to September 2021 [3], whereas the disease name PCM-C appeared 2 years earlier than the fungal name [4]. In 2021, Vilela et al. officially renamed the causative agent as Paracoccidioides cetii based on molecular phylogenetic studies [3]. Interestingly, at least two genotypes have been identified in P. cetii [3]. One of these genotypes is identical to that of P. brasiliensis, P. restrepiensis, and P. venezuelensis, which are causative agents of paracoccidioidomycosis endemic to Latin American countries [1]. Although P. cetii has genomic and morphological similarities such as multiple budding yeast cells in infected host tissue to the causative agents for paracoccidioidomycosis, it is difficult to culture except for animal passages [1, 3].

The transition of disease names corresponds to causative agents. At first, the disease name has appeared as lobomycosis, caused by Loboa loboi which was referred to as Jorge Lobo’s disease, and had been used from 1931 to 1938. Furthermore, the name “lobomycosis” had been used from 1938 to 1998 in humans and in dolphins’ cases. Subsequently, the name “lacaziosis” caused by Lacazia loboi had been applied from 1999 to 2018 regardless of hosts [1, 3].

Recently, the concept of lacaziosis has changed. The cases have been limited to humans and endemic to the Amazonian areas from 2018 to 2021, based on the genetic differences between the causative agents derived from human and cetacean cases. Just at the same time, the disease name in cetaceans was also changed to paracoccidioidomycosis ceti caused by Paracoccidioides brasiliensis var. ceti, between 2016 and 2021 [1, 3]. In addition, L. loboi was renamed as Paracoccidioides loboi in 2021 [3]. Thus, the gene sequences derived from lacaziosis of cetaceans and PCM-C were submitted to the GenBank database as Paracoccidioides spp. [5–7] before the proper settlement of the fungal name.

The host cetacean species of PCM-C include five cetaceans: Atlantic bottlenose dolphin (Tursiops truncatus), Indo-Pacific bottlenose dolphin (Tursiops aduncus), Pacific white-sided dolphin (Lagenorhynchus obliquidens), estuarine dolphin (Sotalia guianensis, also known as the costero dolphin), and Indian Ocean humpback dolphin (Sousa plumbea) [1].

Diagnoses for PCM-C are based on clinical symptoms and detection of typical round yeast cells arranged in chains or producing multiple buddings, but molecular biological, and serological data remain to be auxiliary methods [1, 8, 9]. On the other hand, serological tests have helped in the epidemiology of PCM-C in nursing dolphins in Japan. A survey demonstrated that 61.0% of nursing dolphins showed antibodies against the causative agent [10]. Furthermore, suspected cases of PCM-C showing characteristic cutaneous lesions were observed in 17.1% of the population in the wild Indo-Pacific bottlenose dolphins inhabiting warm waters in Japan (Nagasaki coast, Kyushu area) reported by Van Bressem et al. [11], and there was a stranded case of Indo-Pacific bottlenose dolphin at Kinkou-wan, (Kagoshima, Kyushu area) also reported as a “lobomycosis-like disease” by Tajima et al. [12]. Nevertheless, there has been no survey on the seroprevalence against the causative agent for PCM-C in wild cetaceans.

Cross-reactivity among the causative agents of PCM-C and related fungal species, such as Histoplasma capsulatum, Paracoccidioides brasiliensis sensu lato, Coccidioides spp., and Arthrographis kalrae was necessary to be taken into consideration in the survey. Nevertheless, the survey was successful in nursing dolphins in Far East areas based on the hypothesis that there was an absence of human paracoccidioidomycosis, blastomycosis, and coccidioidomycosis (CCM) in Japan, as well as a lack of cross-reactions between H. capulatum and P. cetii derived from dolphins in the areas [8–10]. However, cross-reactivity due to CCM cannot be ignored in a survey of wild cetaceans because of an autochthonous case of CCM in a boy who developed severe pneumonia after drowning in the South China Sea [13].
According to one of co-authors, there are many stranded dolphins at the seashores of Hokkaido located at the northern area in Japan. Furthermore, there is no information on the PCM-C in dolphins living in cold waters.

These incidences inspired us to investigate the seroprevalence against the causative agents for both PCM-C and CCM simultaneously in wild dolphins; Dall’s porpoises (Phocoenoides dalli) and harbor porpoises (Phocoena phocoena) stranded in Hokkaido, Japan.

Materials and Methods

Serum samples

Two serum samples (from PCM-C cases in Japan) from bottlenose dolphins [5] and Pacific white-sided dolphins [6, 7] infected with *P. cetii* were used as positive controls [8–10]. One serum sample from a healthy person with a medical record of CCM [8, 9] (the usage of human sera was approved by the ethics committee of the University of the Ryukyus, No. 383 approved on November 24, 2017) was used as the positive control.

Serum samples from 15 Dall’s porpoises and 11 harbor porpoises stranded along the coast of Hokkaido, Japan were used. The sex, age, body length, weight of the dolphins were not recorded. Blood samples were collected from cardiac clots from the dead bodies by volunteers. The blood samples were centrifuged at 3,000 rpm (190 G) for 15 min to obtain sera. The use of cetacean serum samples was approved by the Animal Welfare Committee of the University of the Ryukyus (No. 2018079). In addition, there was no evidence of cutaneous abnormalities linked to PCM-C in the dolphins.

Detection of Antibodies

Yeast cells of *P. cetii*; strain SUM having GenBank accession No. AB811031 derived from the skin lesion of the first Japanese case of PCM-C in bottlenose dolphin [5], and various stages of *C. posadasii* [14] (isolate IFM 4935, from a Japanese patient [15]) cells in murine pulmonary tissue embedded in paraffin blocks [16] were used as the antigens [5].

The fungal samples embedded in paraffin blocks were cut into 8 mm thick pieces, placed on poly L-lysine-coated glass slides (S7441; Matsunami Glass Co., Ltd., Osaka), and deparaffinized as previously reported [8–10]. The slides were washed three times with phosphate-buffered saline (PBS) (167–14,491; Wako), and the tissue containing fungal cells were blocked with 100 μl of 5% skim milk (19,810,605; Wako) dissolved in PBS (SM-PBS) for 15 min at room temperature (25 ± 5). After discarding the SM-PBS, 100 μl of diluted sera at 1000-fold, 5000-fold, 10,000-fold, and 50,000-fold in SM-PBS used as the primary antibody were placed on the tissue containing fungal cells in a moistened box. SM-PBS without the primary antibody was used as a negative control. The samples were then incubated at 4 °C for 16 h and washed three times with PBS. The samples were then incubated with the horseradish peroxidase-conjugated rabbit polyclonal anti-dolphin immunoglobulin G antibody (HRP-AD; 100 ml; ab112789; Abcam, Cambridge, UK) diluted 500-fold in PBS for 30 min at room temperature (25 ± 5). In addition, the human serum with a history of CCM was processed as the same procedure as the previous report by Shumoto et al. [8]. All the samples were washed three times with PBS and irradiated with 3,3’-diaminobenzidine (Histofine SAB-PO(M) Kit; Nichirei Biosciences, Tokyo, Japan) for 10 min at room temperature following the manufacturer’s protocol. They were then stained with hematoxylin (131–0966; Wako), sealed with Canadian balsam (192–16,301; Wako), and observed under an optical microscope. We judged the sample as positive when the yeast cells stained brown and titers were greater than 1,000-fold based on our previous report [8].

Statistical Comparisons

Positive rates of the antibodies against *P. cetii* and *C. posadasii* were compared between cetacean species. Statistical comparisons were made by Fischer’s exact probability test (95% significance level, *p* < 0.05), using R ver.4.1.1.

Results

The sera used for seropositive controls against the causative agents for PCM-C and CCM reacted
properly with the same values as in previous reports [8–10] (Fig. 1a–d).

The sera from seven of 15 Dall’s porpoises were positive for *P. cetii* corresponded to 46.7%. The sera from six of the seven dolphins reacted against *P. cetii* at a 1,000-fold dilution alone, and one of the sera reacted positively to both fungal antigens, against *P. cetii* and *C. posadasii*. In addition, the sera from two Dall’s porpoises reacted up to a 5,000-fold dilution against *P. cetii*. In contrast, no serum reacted positively against *P. cetii* in harbor porpoises (Table 1).

The sera from three Dall’s porpoises were positive against *C. posadasii* fungal cells at a 1,000-fold dilution corresponded to 20.0%. In addition, two of them reacted independently against *C. posadasii* without a positive reaction against *P. cetii*. Serum from one of eleven harbor porpoises also showed a positive reaction against *C. posadasii* fungal cells at a 1,000-fold dilution corresponded to 9.1% (Table 1). In addition, there was no dolphin sera which showed positive reaction against *C. posadasii* at a 5,000-fold dilution.

There was a significant difference in the seroprevalence against *P. cetii* between that of Dall’s porpoises (7/15; 46.7%) and of harbor porpoises (0/11; 0%) at 1,000-fold dilution, with an odds rate of 1 vs 20.29 (*p* < 0.01, ranged from 1.01 to 406.36), while there was no significant difference in the seroprevalence against *C. posadasii*. The seropositivity against *C. posadasii* alone, and the simultaneous positivity against both *P. cetii* and *C. posadasii* showed no significant difference between the cetacean species (Table 1).

**Discussion**

The present study demonstrated the antibodies against the causative agent for PCM-C and CCM in wild
cetaceans in the subarctic areas of the Far East. It demonstrated the dolphins have been exposed to the fungal pathogens during their life.

The significantly higher seroprevalence against \textit{P. cetii} in Dall’s porpoises are considered to be attributed to the difference of the habitats as follows; harbour porpoise has a high coastal habitat [17], while Dall’s porpoise inhabiting in a wide distribution at open seas [18], and the latter species has a higher frequency of contact with warm currents resulting in a higher exposure to the causative agents by migration including to the point of confluence of the cold Oyashio Current and the warm Kuroshio Current along the coast of Japan which are located from southern Hokkaido to off Boso (Chiba Prefecture, Kanto, Japan) [19].

The antibodies against \textit{P. cetii} detected in wild Dall’s porpoises stranded at the Hokkaido area indicated that the various dolphin species inhabiting the subarctic region have been exposed to the causative agent, although cases of PCM-C have not been reported from these areas at the present. The information on cutaneous conditions or health examinations of the stranded individuals was lacking in the present study, but it is important to conduct clinical and pathological examinations to find a new case in non-recorded cetacean species.

The most important finding was that the antibodies against \textit{C. posadasii} were detected in two small cetacean species as the first records. Furthermore, there were dolphins in the subarctic area which showed positive seroprevalence against \textit{C. posadasii} and negative results against \textit{P. cetii} independently in two Dall’s porpoises and one harbor porpoise. \textit{Coccidioides} spp. belong to Onygenales, Onygenaceae and closely related to Ajellomyctaceae, including genus \textit{Blastomyces}, \textit{Histoplasma}, \textit{Paracoccidioides}, and \textit{Emergomyces} [20–25]. According to Shumoto et al., the cross-reactions among \textit{P. cetii} and the above fungal genera could be ignored in the non-endemic areas [8, 9], and investigated the survey on the seroprevalence against \textit{P. cetii} in nursed dolphins [10]. Although there was no information of culture and molecular biological data on the case report of a boy who was affected with CCM after drowning in the South China Sea and demonstrated severe pneumonia [13], the present study supported the report. Therefore, the present study should make us revise the concept that The Far East Area is not an endemic area for CCM, and would issue the warning that lives in the area are exposed to the causative agent of CCM from seawater.

On the other hand, some isolates of \textit{Uncinocarpus reesii}, \textit{Chrysosporium queenslandicum}, and \textit{Chrysosporium} spp., which are related species to \textit{Coccidioides} spp. that have been isolated in Japan [26], indicated cross-reactivities between the highly pathogenic fungal genera and above-mentioned species. The isolation of \textit{Chrysosporium} spp. from the marine environment in Korea [27] also cannot be ruled out these cross-reactions. It is impossible to deny the cross-reactions caused by related fungal species at the present.

In conclusion, the present study demonstrated positive seroprevalence against the causative agent for PCM-C and CCM in wild cetaceans in the subarctic areas of the Far East as the first records.

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\begin{table}
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\begin{tabular}{|c|c|c|c|c|c|}
\hline
 & Dall’s porpoise & harbor porpoise & Statistical significance & Odds ratio & Range \\
\hline
Number of animals & 15 & 11 & \ & \ & \\
\hline
\textit{P. cetii} positive & 7 (46.7\%) & 0 (0\%) & \textit{p} < 0.05 & 20.29 & (1.01–406.34) \\
\hline
\textit{C. posadasii} positive & 3 (20.0\%) & 1 (9.1\%) & \textit{NS} & \ & \ \\
\hline
\textit{P. cetii} positive alone & 6 (40.0\%) & \textit{p} < 0.05 & 0 (0\%) & 15.74 & (0.78–316.75) \\
\hline
\textit{C. posadasii} positive alone & 2 (13.3\%) & 1 (9.1\%) & \textit{NS} & \textit{C. posadasii} positive alone & \ \\
\hline
Both positive & 1 (6.25\%) & 0 (0\%) & \textit{NS} & \ & \ \\
\hline
\textit{NS}, not significant
\end{tabular}
\caption{Comparison of the seroprevalence against \textit{P. cetii} and \textit{C. posadasii} between Dall’s porpoise and harbor porpoise}
\end{table}
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**Author’s Contribution** HK managed the experiments, analysis and writing the manuscript. MOM contributed to the collections of the serum samples and managed the background data on the dolphins. AW and RT performed the immune staining and judgements of seroprevalence. TE managed the statistical analyses. MAH participated in the scientific discussions. ENI managed the experimental design and research concept for the worldwide endemics of paracoccidioidomycosis ceti. DAS and KU supervised the project, shared equal responsibilities, and serves as the corresponding authors.

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**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest. All co-authors have agreed to use the present report for a part of doctoral thesis by Hikaru Kanegae.

**Ethical Approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The usage of human sera was permitted by the ethics committee of University of the Ryukyus (No. 383, approved on November 24, 2017).

**Human and Animal Rights** Cetacean serum samples were approved by the Animal Welfare Committee of the University of the Ryukyus (No. 2018079). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The use of human sera was approved by the ethics committee of the University of the Ryukyus (No. 383, approved on November 24, 2017).

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