Studies on Paracoccidioidomycosis ceti
(Summary of a Ph.D's thesis)

Tomoko Minakawa, D. V. M.
(Supervisor: Professor Shinpei Wada)

Zoonosis, that is also called zoonotic diseases which is infectious disease that transmitted between human and animals, both wild and domestic. According to Taylor *et al.*, 1415 species of infectious organisms known to be pathogenic to humans. Out of these 1415 species, 868 species (61.3%) are zoonotic and 175 pathogens are relating to emerging diseases. Aquarium have many different kinds of animals, for example fish, reptiles, amphibians, avian and marine mammals. The zoonotic pathogens of these aquatic animal species are different from those in terrestrial animals. Therefore it is essential to know the details of the pathogens to keep these animals healthy. Also it is important to control the sick animals to prevent the disease from spreading for other animals and people. Recently, many cases of fungal transmission between animals and humans are reported.

In this study, I report about the new knowledge about the paracoccidioidomycosis ceti (PCM-C) regarded as a candidate of zoonotic disease, and I represent this new information to keep healthy for Japanese people as marine nation.

In chapter II, the first report of a PCM-C case in Pacific white-sided dolphin (abbreviate to PWSD, *Lagenorhynchus obliquidens*) and a suspected case showed similar symptom in the same kinds of dolphin are described. It includes the detail of clinical symptoms of the dolphins, and the results of clinical pathological, microbilogical, histopathological and molecular biological examinations.

The characteristics of PCM-C formerly called as “lacaziosis” known as “Jorge Lobo’s disease” or keloidal blastomycosis or “lobomycosis” both in humans and dolphins. Taborda *et al.* (1999) classified the causative agent for

“lacaziosis” as *Lacazia loboi* , that is one of the emerging diseases. and the name of “lacaziosis” has been prioritized both in human and cetacean cases by Vilela *et al.* since 2005. Namely, there was no classification of lacazioses both in humans and dolphins at that time. The endemic areas of lacaziosis (paracoccidioidomycosis) are Latin America countries including the Pacific Rim.

However, the term “Paracoccidioidomycosis ceti” (PCM-C) has proposed by Vilela *et al.* (2016), recently. They re-designated lacaziosis in cetacean species as PCM-C caused by uncultured *Paracoccidioides brasiliensis* based on the phylogenetic analyses of multiple genes, and is endemic globally, including in Japan. On the other hand, they defined “lacaziosis” as an endemic fungal disease in Amazonia caused by *L. loboi* in limited to humans. Formerly, lacaziosis was thought to be one of zoonotic fungal infections because of one human case of dolphin trainer transmitted from infected animal, however, it was impossible to diagnose as lacaziosis or PCM-C because of lack of molecular biological data of the causative agent. In Japan, it has been known that the confirmed cetacean host of PCM-C might be a bottlenose dolphin (BD, *Tursiops truncates*), however, many other kinds of cetacean species have been reported as suspected PCM-C hosts.

Case 1 is the first report of a PCM-C case caused by *P. brasiliensis sensu stricto* (Turissini *et al*., 2017) in PWSD. The dolphin was female estimated to be more than 14 years old at the end of June 2015, she was captured off the Japan Sea coast in 2001. The body weight was 79.8 kg in January 2015.

Since the beginning of 2010, the dolphin has shown slight erosive dermatitis on the left peduncle, and has had repeated aggressions and remissions until the apparent manifestation of the granulomatous lesions on the skin in July 2014.

The multiple, lobose, and solid granulomatous lesions, with or without ulcers, appeared on the jaw, back, flipper, and fluke skin in July 2014. The lesions ranged in size from 1 to 2 cm, and up to more than 10 cm in diameter. In January 2015, we took a chest radiography, and found at least 2 bulla-like structures measured from 0.5 to 1.0 cm in diameters.

In case 2, the dolphin (PWSD) was captured off the Japan Sea coast in 1996, and estimated to be more than 25 years old male at the end of August 2016. The body weight was 121.0 kg at April 2016. as been showing multiple lesions of grey-whitish colored dermal tubercles which were similar to those in case 1, or in nontuberculous mycobacterial infection in other cetacean species on the tip of right side of the tail fluke since September 2008. The lesions have gradually increased, fused each other, forming keloidal appearance and covered the most area of tail fluke, right side of the body and ventral side of the keel as well, by August 2016. The individual foci ranged from 1 to 2 cm in diameter, however, the major axes of some fused lesions were up to 15 cm.

These dolphins received routine antibiotics and typical treatment for general dermatological disorders, however, no apparent clinical response was recorded. Some biopsied samples were collected from those dolphins under infiltration anesthesia by lidocaine hydrochloride supplemented with 2 % adrenaline. Those samples were used for clinical pathological, microbiological, and histopathological examinations for fungi and nontuberculous mycobacteria. In addition, molecular biological detections of specific 43-kDa glycoprotein (*gp*43) coding gene

and cytological examinations were also performed with these samples. As a result, fungal elements consisting of chains of spherical budding yeast-like structures ranged from 15 to 25 micrometer in diameters, stained positively by periodic acid Schiff's reaction (PAS) and Gomori's methenamine silver-nitrate (GMS) were detected in cytological samples of Case 1 and Case 2.

On case 1, the partial sequence of *gp*43 gene confirmed by a nested PCR and sequencing, which revealed a different genotype from both Amazonian and Japanese lacaziosis in bottlenose dolphins, and was 99% identical to those derived from *Paracoccidioides brasiliensis sensu stricto*, the latest description of fungal species in the genus *Paracocidioides* .

In chapter III, I describe about the epidemiological survey of PCM-C using immunohistochemistry (IHC) examination with sera obtained from some cetacean species kept in Japanese aquaria, that aim to establish one of the method to diagnose PCM-C. Serum samples from 3 cetacean cases in Japan diagnosed as PCM-C followed to the new classification by Vilela *et al.* (2016) were used as positive control; 2 caused by *Paracoccidioides* sp. in BDs (Case Ⅰ and Ⅱ) and 1 by *P. brasiliensis* in a PWSD (case Ⅲ). The numbers of sera from PCM-C recorded cetacean species were 31 dolphins. The non-recorded dolphins were 10 animals.

Infected tissue derived from case Ⅰ was fixed with 10% formalin and stored at room temperature. After embedding in paraffin wax, the samples were cut into 8μm sections, and placed on poly-L-lysine-coated glass slides. After that the slide was deparaffinized for IHC. Skim milk diluted to 5% with phosphate-buffered saline was used as the negative control.

Statistical analysis for the comparison between sera from PCM-C recorded and non-recorded species, among cetacean species, and among aquaria with or without history of PCM-C were evaluated by Pearson's chi-square method with Odds ratio and two tailed Fischer's exact probability test *via* the website: http://vassarstats.net/odds2x2.html.

There was species dependent significance in the prevalence ratio between BDs and Indian Ocean bottlenose dolphins (IOBDs, *Trusiops aductus*), and between IOBDs and PWSDs. On the other hand, the relationship between the record of PCM-C and the prevalence ratio of anti PCM-C in aquaria was unclear. For example, the aquarium B having a case of PCM-C showed a significantly lower prevalence ratio compared to the aquarium A at 5,000-folds of dilution. It suggested that a higher prevalence ratio of antibody against to PCM-C might prevent developing the fungal infection represented as aquarium A. On the other hand, an equivalent significance was also detected between aquarium C and aquarium A without record of PCM-C. There were no conclusions on the relationship between PCM-C record and prevalence ratio in housemates, at the present. In this chapter, it was revealed that at least 61.0% of cetaceans nursing at aquaria in Japan possessed antibodies positively reacting to PCM-C antigen.

In the recent studies, the author could conclude that the pathogen of PCM-C has already existed in the Japanese coastal water, and Japanese aquatic veterinarians should prepare for the occurrence of this condition in the near future. More detailed studies including establishment of accurate diagnostic molecular and/or IHC methods for PCM-C. Free ranging cetaceans, captured

or stranded around Japanese coastal area, should be the next preferable targets for the studies of PCM-C.