Algological study of the genus Prototheca

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Prototheca was first reported by Kruger in 1894 to be nonpigmented unicellular organisms isolated from the slime flux of trees. They were considered to be yeast because their colony were similar to that of yeast, and classified into 2 species, Prototheca zopfii and P. moriformis. In 1916, Prototheca was reclassified as algae because these spore is internally in multiplication identical to that of Chlorella. Number of studies related with the difference between the genus Prototheca and Chlorella, had been investigated and it is now generally considered that the genus Prototheca developed from Chlorella at some point in mutation.

Prototheca have been isolated from slime flux of trees, feces in animals and human, potato skin, stream water, sludges in waste stabilization pond, diseased tissue associated with animal and human.

The investigations of Prototheca concerning to

classification have been performed in view points of morphology, physiology, biochemistory and serology. From these results, the species of Prototheca were classified as 7 species(P. zopfii, P. moriformis, P. portoricensis, P. ciferii, P. wickerhamii, P. segbwema, P. stagnora) in 1968, 5 species(P. filamenta, P. zopfii, P. wickerhamii, P. moriformis, P. stagnora) in 1972, and 3 species(P. zopfii, P. wickerhamii, P. stagnora) in 1975. However, Pore, R.S. reported in 1983 that the Prototheca species reclassified into 4 species(P. zopfii, P. moriformis, P. wickerhamii, P. stagnora) from the result of morphological and physiological characteristics).

As above describe, the consensus of opinion concerned with the classification of Prototheca is not established in present.

This study dealed with morphological, physiological, biochemical serological characteristics for the classification of Prototheca species.

Protothecosis is included the opportunistic infection as well as almost of mycoses. There are now various types infections being caused by Prototheca species. Protothecosis have been reported in cattle, dog, deer, fish and human.

Especially, many cases have been found in bovine mastitis and disseminated disease of dog. Therefore, Prototheco sis is an important disease in veterinary medicine. However, there are a few reports concerning with the pathogenicity of Prototheca inspite of many cases have been reported. Moreover, there is a small number of study concerning to sensitivity against antimicrobial agents.

In this study, the experimental infection in mice and in vitro phagocytosis using peritoneal exudate cells were examined. Moreover, the comparison study of sensitivity to several therapeutic agents against Prototheca is investigated.

These results in this study were described as follows;

1. Ecological study of the genus Prototheca in nature.

It is a fact that Prototheca is isolated from natural sources, yet there has been no systemic investigation into the ecology of Prototheca, especially in Japan. An ecological study is one of importance for several reasons. Sabouraud's dextrose agar, Prototheca isolation medium(PIM) and modified

PIM(mPIM) were used for isolation of Prototheca from natural sources. The isolation rates in PIM and mPIM were superior to its in SDA. Namely, the organisms are isolated from 10(SDA), 30(PIM) and 40%(mPIM) in pond; 2, 10 and 14% in slime flux of trees; 16, 42 and 50% in stream water; 0, 4 and 8% in soil, but not from 572 samples of milks.

2. Algological characteristics of Prototheca.

Prototheca species are microscopic, achlorophyllos single-celled organisms whose a life cycle(Autospore formation) similar to that of the genus Chlorella. Maturely single-cell(mother cell) produce internally several endospores(daughter cell). Thus, the life cycle and morphology of Prototheca species were examined by means of light microscopy, scanning electron microscopy and transmission microscopy.

In light microscopic finding, the mother cell of P. zopfii 7918-1, 8008-1, B-1270 isolated from lesions were ellipsoidall, but that of P. zopfii B-1266 was ellissoidall-globose ,and P. wickerhamii and P. stagnora were globose. It was characterization that in comparison of daughter cell, the sharp and number ware variable between species, within

species and within strain.

All Prototheca species showed smooth surface by the observation using scanning electron microscopy. The cell wall of mother cell was east to rupture. The sharp of Prototheca species were identical to the observation of light microscopy.

That is, the differences of sharp were observed within species, i.e. 7918-1, 8008-1, B-1270 isolated from lesion were large-ellisoidall, while B-1266 isolated from nature was small ellipsoidall-globose.

In transmission electron microscopic finding, nucleus, granular material and mitochondrion were observed and the ultra structural feature was that the cell wall was composed of two layers.

Eight strain were examined for cultural characteristics. All of them were found to be achlorophyllous, multiply by autospore formation, inhibited growth by cycloheximide and stimulated growth by vitamin B1. They grew well on SDA and their colonies were identical to that of yeast. The colony color in P. zopfii changed from white to brown-yellow during long time incubation. Of P. wickerhamii, B-1421 showed granule-like colony in

different of B-1280 produced cream colony.

In growth temperature, 7918-1, 8008-1 and B-1270 grew on SDA ranged at 15 to 42 °C, but B-1266 not at 36 °C. For P. wickerhamii, B-1280 grew at 15 to 42 °C, but B-1421 was inhibited the growth at 15 and 42 °C.

The assimilation of 19 carbon sources in Prototheca species was examined by using API 20C system. All strains assimilated glucose as carbon source. Galactose was utilized by P. zopfii and P. wickerhamii. Treharose was assimilated by only P. wickerhamii. Prototheca was classified into 3 species by using API 20C.

The enzymatic profile of Prototheca species was examined using the API ZYM system. P. zopfii 7918-1, 8008-1 and B-1270 showed the same enzymatic profile. While the other strains were difference in enzymatic profile within species and strains. Namely, esterase(C4),esterase lipase(C8), leucine arylamidase, phosphatase acid, phosphoamidase and minimal activities of phosphatase alkaline, lipase(C14) were detected from 7918-1, 8008-1 and B-1270; esterase(C4), esterase lipase(C8), lipase(C14), leucine arylamidase and minimal activities of phosphatase alkaline, valine

arylamidase, phosphatase acid, phosphoamidase were detected from B-1266; phosphatase alkaline, esterase(C4), esterase lipase(C8), leucine arylamidase, phosphatase acid, phosphoamidase and minimal activities of trypsin were detected from B-1421; esterase(C4), Esterase lipase(C8), phosphatase acid, phosphoamidase and minimal activities of phosphatase alkaline, leucine arylamidase, trypsin were detected from B-1280; esterase(C4), phosphatase acid,phosphoamidase and minimal activities of phosphatase alkaline, esterase lipase(C8), trypsin were demonstrated from B-1277.

None of the other enzymes(Cystine arylamidase, chymotrypsin, α-galactosidase, β-galactosidase, β-glucronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase) were demonstrated in any species.

from this result, the API zym system was non-useful for the identification of Prototheca species, but useful for the judgment of the genus.

Quantitative protein and sugars which composed of Prototheca were no significant difference between strains except for B-1421 and B-1270. Quantities of protein and sugar were 45 to 80 mg/dl and 0.2 to 0.4 mg/dl, respectively.

In comparison of sugar using thin layer chromatography with naphtorezocinol-sulfate, 7918-1, 8008-1 B-1270 and B-1277 were found identical 9 spots, while B-1266, B-1421, B-1280 were 2 spots. In comparison of sugar with iodine, 7918-1, 8008-1, B-1270 and B-1277 were detected 5 spots, B-1266 was 2 spots, B-1421 and B-1280 were 2 spots.

In comparison of amino acid, Rf values were detected nine in 7918-1, 8008-1,B-1270 and B-1277, five in B-1266, four in B-1421, and eight in B-1280.

In comparison of lipid, Rf values were two in 7918-1, 8008-1, B-1270, B-1277 and one in B-1266, B-1421, B-1280. The constituents of sugar, amino acid and lipid were recognized significant difference within strains except for 7918-1, 8008-1 and B-1270.

The serological relationship of Prototheca strains was investigated by gel immunodiffusion and indirect immunofluorescence tests. The antisera obtained from bovine mastitis with Prototheca reacted with homologous antigen(7918-1, 8008-1) and antigen of B-1270. On the other hand, antisera against B-1266, B-1421 and B-1277 reacted with homologous

antigen, but remaining antisera not only with homologous but also with heterologeous antigens. Antiserum of B-1270 reacted with homologous antigen and heterologous antigens of 7918-1 and 8008-1, but not with the other antigens.
The reaction of B-1270 was identical to that of 7918-1 and 8008-1. These strains examined were designated into 5 serotypes by gel immuno diffusion test.

In indirect immunofluorescence test, antiserum of diseased cow reacted with all antigens except for B-1280 and B-1277. On the other, antisera made with formalized antigen reacted not only with homologous antigen but also with heterologous antigens. Namely, antisera of 7918-1, 8008-1 B-1270 and B-1280 reacted with all of heterologous antigen ,and its of B-1266, B-1280 and B-1277 reacted with B-1421. Thus, antisera were absorbed with each of heterologous antigens. Specific antisera reacting with only homologous antigen were obtained in B-1266, B-1421,B-1280 and B-1277, respectively. However, the reactions of 7918-1 and 8008-1 were completely absorbed with antigen of B-1270. Like this, these strains were designated 5 serotypes as well as result of gel immunodiffusion test.

3. Pathogenicity of Prototheca.

This study was designated to clarify the virulence of Prototheca species for mice, the method for production of experimental protothecosis and the defense mechanism against protothecosis by peritoneal exudate cells in vitro. Comparison was made on the virulence of Prototheca species for mice by calculating LD_{50} value. None mice injected with 10^{10} cells died. Therefore, mice were pretreated with predonine(1 mg/Kg) before infection. The LD_{50} of strain derived from lesion was $10^{7.5}$ cells, whereas that of strain derived from nature was $10^{8.5}$ cells. It was proved that virulence and avirulence strain existed within Prototheca species and mice should be pretreated with predonine for production of experimental protothecosis.

Multiplication of organisms in mice pretreated with predonine, nitrogen mustard, predonine plus nitrogen mustard or not were compared with number of viable counts in organs. The organisms of organs in mice pretreated with predonine or predonine plus nitrogen mustard were isolated more than 10^2 to 10^6 in compared with its in mice pretreated with

nitrogen mustard or not. This result revealed that P. zopfii was inhibited by the reticuoendothelial system.

The viable counts of organs in mice pretreated with predonine or predonine plus nitrogen mustard were $10^{4.46}$ to $10^{7.5}$ cells/g. In mice pretreated with nitrogen mustard or not, the organisms were isolated $10^{3.46}$ to $10^{5.20}$ cells/g from kidney, $10^{0.97}$ to $10^{3.37}$ cells/g from brain and $10^{0.60}$ to $10^{0.70}$ cells/g from lung, respectively. From these results, it was proved that the target organs were kidney and lung as aspergillosis and candidiasis when it intravenously invaded, and the organisms survived for long time in kidney and brain.

Histopathologically, the lesion was identical to the result of viable counts. Namely, mainly lesion were found in kidney.

The relationship between Prototheca and mouse peritoneal exudate cell was examined to clarify the defense mechanism of phagocytosis in vitro. There were no significant differences in the rate of phagocytosis and extracellular viable counts between polymorphonuclear cell(PMN) and macrophage, whereas a significant difference in the intracellular viable counts($P<0.01$). That is, the intracel-

lular viable counts in macrophage decreased after 2 hr., while its in PMN increased. It was demonstrated that the important phagocytes in defense mechanisms was not PMN but macrophage.

4. In vitro antiprotothecal activity of drugs.

The protothecosis indicated various types from surface to disseminated infection. However, there is a few reports concerning to therapeutics, since detailed investigation related with sensitivity test against Prototheca was not performed. Thus a total of 20 antibacterial and antifungal drugs were examined to clarify the antiprotothecal activities. The MICs of streptomycin and kanamycin were 50 mcg/ml for Prototheca species. The growth of all strains were not inhibited with the other drugs. tetracycline had MIC of 50 mcg/ml for only B-1266 and B-1277.

Prototheca had sensitivity to antifungal agents except for griseofulvin, trinaftate and 5-fluorocytocine indicating MICs of more than 100 mcg/ml. The MICs of antifungal agents for Prototheca species ranged from 0.8 to 3.2 mcg/ml in amphotericin B and nanaomycin, 1.6 to 6.25 mcg/ml in miconazole, 6.25 to 25 mcg/ml in batrafen, 12.5

to 25 mcg/ml in CN-146 and 6.25 to 50 mcg/ml in ketoconazole, respectively. The MIC of clotrimazole ranged from 3.2 to 6.25 mcg/ml for the other strains except for 7918-1, 8008-1 and B-1270. The MICs of strains derived from lesion indicated a resistance of ranging from twofold to fourfold in compared with these of strains derived from nature.

From the results of this study, it was revealed the conclusions as follows,
1) Ecology of Prototheca, 2) Genus Prototheca is able to classify into 3 species(P. zopfii, P. wickerhamii and P. stagnora) by simple method of API 20C system. 3) Genus Prototheca is able to distinguish 5 serotypes by gel immunodiffusion and indirect immunofluorescence tests. 4) In vitro defense mechanism of phagocytosis against Prototheca with peritoneal exudate cells. 5) In vitro effective antiprotothecal drugs.