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Identification, Pathogenicity and Toxicity of *Absidia* sp. isolated from Cattle

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Recently, deep or systemic mycosis in human beings and animals has been receiving more attention year to year, because of the difficulty of its diagnosis and the increasing recognition of cases. Nevertheless, the antigen-antibody test of animal mycosis has made some progress in some animal species[33], and vaccination to some domestic animals has been also applied for the therapy of the mycosis[25]. Sasaki[34] described that numerous fungi caused the mycosis in animals and that the main causal fungi were as follows; *Aspergillus*, *Mucor*, *Absidia*, *Rhizopus*, *Mortiellera* and *Candida*. Infectious diseases in animals caused by these fungi have been studied intensively by foreign workers, particularly by Ainsworth and Austwick[1,2], Hillman[15,16], Corbel et al[5~7], Cordes et al[8] and Hunter et al[17], but are limited in number reported in Japan, except the diseases of chickens[12,19], turkys[40] and ruminants [18,22,23,30,36,39,46]. The present authors have already reported bovine mycotic abortion caused by *Aspergillus fumigatus* and *Mucor pusillus*[22,23]. Among various kinds of bovine deep mycosis, bovine gastritis cases caused by fungal infection have been also reported by some investigators in foreign countries[1,9,11,13,27,28,31] and Japan[18,30,36]. The causal fungi have been noted by those authors listed as above. The main fungi are *Aspergillus fumigatus*, *A. niger*, *A.* spp. and *Absidia ramosa*, *A.* spp., *Rhizopus cohnii*, *Mucor* spp. and *Candida* spp. These are ubiquitous and non-pathogenic to animals under normal and well-managed conditions.

This investigation chiefly deals with the identification of one of the fungi isolated from a lesion in the omasum of a cow among the ten which were brought to the Veterinary Pathological Laboratory in the College of Dairying from January to December in 1979 for pathological investigation. The pathogenicity of the fungus to mice was also examined by intravenous and intraperitoneal injections of the spore suspension and concentrated fungal

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cultural filtrate, respectively. We also examined the number of the fungi in feeds, litter and hay in a barn in which the history of the mycosis in cattle was recorded.

Materials and methods

Cattle cases Ten cattle cases were submitted to the Veterinary Pathological Laboratory from January to December in 1979 for pathological and mycological investigation. One case among ten was Japanese black breed, and the nine cases were Holstein breed, shown in Table 1. All the materials were subjected to necropsy as soon as they were brought in.

Table 1. Cattle cases from which Mucorales were isolated

Case No.	Breed	Organs from which *Ab. corymbifera* were isolated	Isolated fungi
Pr 286	Wagyu	Lung, Kidney, Spleen, Liver	*Mucor pusillus*
Pr 297	Holstein	Rumen, Reticulum	*A. corymbifera*
E 2607	〃	Placenta (mother)	*A. corymbifera*
Pr 356	〃	Omasum	*Mucor pusillus* *A. corymbifera*
E 2640	〃	Lung, Rumen, Omasum, Abomasum	*A. corymbifera*
Pr 364	〃	Omasum	*Rhizopus* sp.
		Rumen, Reticulum, Omasum	*A. corymbifera*
Pr 375	〃	Reticulum, Colon	*Mucor pusillus*
		Rumen, Reticulum, Colon	*A. corymbifera*
E 2687	〃	Placenta (fetus), Blood	*A. corymbifera*
E 2717	〃	Rumen, Omasum, Abomasum	*A. corymbifera* *Rhizopus* sp.
Pr 402	〃	Rumen	*Rhizopus* sp.

Isolation of fungi The lesions which were suspected to suffer from the mycosis were immediately severed after necropsy and immersed in 0.1% mercuric chloride solution for thirty seconds, respectively, in order to sterilize the surface of each tissue. The tissues were rinsed twice with sterilized distilled water, respectively. They were respectively placed in another sterilized Petri dishes and were cut into smaller pieces with a sterilized forceps

and knife. Malt extract agar[45] containing 0.03% of chloramphenicol succinate (Sankyo Seiyaku Co.) to inhibit the growth of bacteria was melted, then poured into the dishes and solidified. All the cultural plates were incubated at 37°C for two days, and all the fungal colonies grown on the tissues samples in the plates were respectively transferred on each slant of the medium.

Identification of fungi Synthetic mucor agar medium[45] was generally used for the identification of the fungus which was predominantly isolated in number. The fungus grown on the medium was observed by giant colony, slide culture and ordinary microscopic technique in detail of the morphological aspects. The fungus was examined in effect on growth to various degrees of osmosis using NaCl and sucrose (5 to 40% in 5% interval) and pH values (2 to 10 in 1 interval), using malt extract agar for the test of osmosis and liquid malt extract medium for the test of pH. Casein nutrient agar and Crossley's victoria blue margarine fat nutrient agar (Eiken Co.) were used for the detection of protease and lipase excreted by the fungus, respectively. The effect of the different degrees of temperature on the fungal growth was examined from 10° to 80°C in 5°C interval. The identification of the fungus was referred to Ainsworth, Sparrow and Sussman[3,4], Lendner[20], Zycha, Siepmann and Linnemann[47], Hesseltine and Ellis[14], Udagawa et al[44]. The fungus was also compared with *Absidia corymbifera* IFO 4010.

Pathogenicity of the fungus to mice A test for the fungal pathogenicity was applied to ten weeks old d-d Y mice purchased at Hokkaido Animals Experimental Center. The test was to examine whether the inoculation of the fungal spore suspension or the fungal metabolites would cause mycotic infection and show toxicity. The five of the ten mice were intravenously inoculated with 2.0 ml of the spore suspension, respectively, of which the preparation procedure will be described later. These mice were compared to the mice injected with sterilized physiological saline solution in the same volume as above. The preparation of the spore suspension was as follows; the preserved fungal culture on malt extract agar slant was inoculated on malt extract agar plates. After the plates were incubated at 37°C for ten days, the spores were collected, suspended in the solution, and adjusted to 10^8/ml in spore number. The first inoculation into mice was carried out at the end of the year when the fungi were isolated, before the pathogenicity was lost or weakened by successive culture on synthetic culture medium. The second into the surviving mice took place on the 10th day after the first one.

The second test was to examine whether the cultural filtrate would be toxic to mice or not. The cultural filtrate was prepared as follows; the preserved fungal culture was inoculated in liquid malt extract medium. Two

hundred ml of the medium had been previously poured into a 500 ml Elrenmyer flask with a cotten plug and was autoclaved at 121°C for 10 minutes. These ten flasks inoculated with the fungus were incubated at 37°C for ten days. Then, the mycelia and the spores were excluded through filter paper and Seitz filter, and the filtrate was concentrated to 1/20 in quantity under 16 mm in Hg at 60°C. 0.5 ml of the concentrate was intraperitoneally injected into each of the five mice. In order to prepare another five control mice, the concentrated liquid malt extract medium in which the fungus had not been inoculated was prepared by the same method described above, and 0.5 ml of the concentrate was intraperitoneally injected to the five mice, respectively. The first and second injections of the concentrate or concentrated medium took place on the same days as in the inoculation of the spore suspension.

As soon as the mice to which the spore suspension was inoculated were dead, these were examined in the brain, kidney, heart, liver, lung and spleen. The surviving and control mice were killed on the 31st day after the first inoculation and were examined as the dead mice were. Portions of these organs were subjected to mycological and histopathological examinations, except for the cases injected with the cultural filtrate. The method of fungal isolation was the same as the one in the cattle cases.

Histopathological examination in cattle and mice For the cattle, the lesions which were suspected to suffer from the mycosis were excised. For the mice which were inoculated with the spore suspension and physiological saline solution, the six organs mentioned above were removed. Half of each organ from each mouse was submitted to isolation of the fungus and the rest of half of each organ was used for histopathological examination. In the histopathological examination, each material of both cattle and mice was fixed in formalin, embedded in paraffin after dehydration in alcohol, sectioned approximately 5 μm thick, then stained by H-E and PAS using the same methods as in the previous papers[22,23]. Histopathological observation was made on each tissue-section sample.

Colony count of fungi in a barn The number of the colonies of aspergilli, Mucorales excluding the number of the colonies of *Absidia corymbifera* and *Absidia corymbifera* was counted from animal feeds and litter of rice straws in a barn in which a deep mycosis case had broken out. Materials for fungal colony count were as follows ; one kind of hay (A) examined in this experiment had been stored in the barn, and the other hay (B) had been stored in an outside hay barn and was more moist in appearance than the former. Table 2. A kind of silage (A) which was exposed to air for a half day in the barn after removal from a silo, and the other one (B) was kept in the silo until it was removed for the experiment. In the colony

count of litter, one type of litter (A) was stored in a litter barn, and the other (B) was kept outside and wrapped in a vinyl cover and appeared more moist than the former. The animal feed used for the experiment was a type of formula feed. Beept pulp was crushed and exposed to air for a half day in the barn. The procedures for the colony count for each material were the same as those in the previous paper[22].

Results

A deep mycosis case and other cases The ten cattle cases which were submitted to necropsy were diagnosed as not suffering from real deep mycosis with the exception of a single case. The nine cases were afflicted with other diseases. The details in pathological observation of each case will be reported in another paper. Among the cases, one case (E 2640) was died of lymphosarcoma and was also diagnosed as suffering from mucormycosis in the omasum and abomasum. Many hyphae of a fungus belonging to Mucorales which were big in diameter and did not possess septa were observed in those organs histopathologically. In the other nine cases, three kinds of fungal genera were isolated from the lesions in various organs and blood (Table 1). The genera were belonging to *Mucor*, *Absidia* and *Rhizopus*. *Absidia* sp. was predominant in the number of all the isolates.

Identification of the fungus isolated in the most predominant number The morphological characteristics of the fungus, *Absidia* sp. which was isolated from the omasum of E 2640 cattle case were as follows ; the giant colony on a synthetic mucor agar plate at 37°C grew rapidly, attaining more than 2 cm in height, and filled the plate of 9 cm in diameter within four days. The aerial mycelia in the central part grew more rapidly, compared with the mycelia in the colonial margin. The mycelia appeared as white color at the beginning, then became silver grey when matured. No pigmentation and zoonation were recognized (Fig. 1). Mycelia : These were colorless, not septated, and 10～15 μm in diameter. Branched rhizoids were not formed directly underneath sporangiophores. Sporangiophores : These were corymbously ramified but were not circinated and were formed columellae at each apex. The color was trasparent at the beginning and was tinged with light brown when matured. The diameter was 5～6 μm (Fig. 2). Columellae : These were formed at each end of sporangiophores as swelling in globose or hemispherical shape, and were colorless or light brown. The diameter was 10～20 μm (Fig. 3). Sporangia : These were formed directly from the sporangiophores. The surface of the membranes was smooth at the beginning but became rough, and the membranes were dissolved at the end of growth, remaining frills at the base. The size was varied from 20～80 μm. Each corymbous branch in the lower part of sporangiophores sometimes

formed smaller sporangium at the tip. The average size was 45～60 μm (Fig. 4). Spores: These were spherical or oval in shape, and colorless. The surface was smooth. The diameter was 2.5～3.5 μm (Fig. 5). Zygospores: These were not formed. Physiological characteristics of the fungus were as follows: The fungus grew on the media, containing 5% of sodium chloride and 40% sucrose, respectively and grew between pH 2.0～10.0. Protease and lipase excreted from the fungus were confirmed. The range of growth temperature was between 20°C and 50°C, and the optimum growth temperature was 40°C. According to these characteristics of the fungus, it was identified as *Absidia corymbifera*.

Fungal pathogenicity and toxicity to mice Three mice among the five into which the spore suspension was intravenously inoculated succumbed from the third to fifth day from the date of the first inoculation. The other two mice were debilitated and lived on until these were sacrificed, in spite of two times inoculations. At the necropsy of all the five mice, liver, kidney and spleen were cloudy, and showed hemorrhage and whitish-grey nodules. From all the five organs, the fungus was isolated and was identified as the same fungus as the inoculated one. In histopathological examination, the hyphae which were presumably assumed as the hyphae of the inoculated fungus were recognized in the tissue specimens of the kidney and liver (Fig. 6). All the five mice into which the fungal metabolites were intraperitoneally injected survived until they were sacrificed. In all these cases, cloudiness and

Table 2. The number of the colonies of various animals feeds in a barn at 37°C

fungi / samples	Aspergilli	Mucorales	Yeasts	*Absidia corymbifera*
Hay A	3.6×10^2～8.0×10^2	6.0×10^2～2.0×10^3	10	6.0×10～2.0×10^3
Hay B	2.0×10^4～2.4×10^4	2.0×10^4～3.0×10^4	10	1.0×10^3～4.0×10^4
Silage A	1.0×10^2～6.0×10^4	6.0×10^2～1.4×10^2	1.7×10^5～1.2×10^7	$<3.0\times10$
Silage B	2.0×10	<10	<10	<10
Litter A	<10	5.4×10^3～3.8×10^4	1.2×10^7～2.0×10^7	1.6×10^4～2.0×10^4
Litter B	6.2×10^3～1.8×10^4	6.8×10^3～1.0×10^4	<10	<10
Formula feeds	2.0×10	1.0×10^2	<10	<10～1.0×10^2
Beet pulp	<10	<10	<10	<10

Hay A: Hay in a barn.
Hay B: Hay stored in a hay barn.
Silage A: Silage in a barn.
Silage B: Silage in a silo.
Litter A: Litter stored in a litter barn.
Litter B: Litter kept outside and wrapped in a vinyl cover.
The number of the colonies shows the one per gram of each material.

hemorrhage in the liver, kidney and spleen, either both signs or one was recognized. However, the intensity of the clinical signs was lesser than the cases of the spore suspension inoculation. On the other hand, in the control mice, no significant pathological changes were found, and none of fungi was isolated.

Colony count of fungi in a barn Regarding the experiment of the distribution of fungi in the animal feeds and litter in the barn, the results were shown in Table 2. In the number of the colonies per gram of each material, aspergilli, Mucorales and *A. corymbifera* in the hay (B) were in 10^4, 10^4 and $10^{3\sim4}$, respectively, more than the hay (A). The number of the former two kinds of fungi in the silage (A) was rather larger in $10^{2\sim4}$ and 10^2, respectively, compared with the one in the silage (B), and also a great number of yeasts was counted. *A. corymbifera* was very small in number. In the silage (B), only a few colonies of aspergilli were counted. Mucorales, yeasts and *A. corymbifera* in the litter (A) were counted in $10^{3\sim4}$, 10^7 and 10^4, respectively but aspergilli were not detected. In the litter (B), aspergilli and Mucorales were counted in both $10^{3\sim4}$ and the other two of the fungi were not found. In thə formula feeds and beet pulp, the number was negligible.

Discussion

In recent years, cattle are daily supplied by a great amount of concentrated feeds without a sufficient quantity of roughage, not taking into account the characteristics of those which are herbivorous. Therefore, it has been reported[24,42] that the mucous membranes of the forestomachs and abomasums of cattle have become afflicted by acidosis. Furthermore, it seems that cattle breeding aimed only at the improvement in milk production and fattening has not been considered in the health of cattle. In addition, it has been described[26,37,41,43] that many antibiotics and corticosteroides have been applied for therapy of bacterial infectious diseases and that it has consequently caused the induction of mycosis.

Marcato et al[21] stated that 59 cattle among 461 in 18 to 24 months which appeared to be healthy had mycotic granulomas in the bronchial lymph nodes and that many kinds of fungi were isolated from the lesions, although it was not clear whether the factors described above would induce the mycosis. Therefore, it may well be speculated that many cattle which were appeared healthy might have lesions of deep mycosis in some organs.

In this fungal colony count in a barn, it was clarified that aspergilli, yeasts and fungi belonging to Mucorales including *A. corymbifera* were predominant in the hay and animal feeds and grew very rapidly by exposure to air within a short period and storage in moist conditions. However, the number was not so numerous as to induce mycosis in healthy cattle under

normal conditions, compared with the number carried out in the previous investigation[22], although the number and kinds of fungi distributed in the barn might be changed various conditions.

It is well known that *A. corymbifera*, *A. fumigatus*, *Mucor pusillus* and *Rhizopus* sp. which were isolated in the experiment were pathogenic to many kinds of animals, and they induced many kinds of diseases under certain conditions. Among these fungi, it has been described that *A. corymbifera* caused abortion in cattle[29,35,38], showed a greater response to bovine placental extract *in vitro* than any other fungi[10] and had a high pathogenicity to mice[7].

However, it has not been recorded in Japan that this fungus causes bovine deep mycosis, that it is one of the main fungi isolated from the mycotic lesions of cattle and that it is distributed in a large number in a barn around the central part of Hokkaido. It might be speculated that *A. corymbifera* would play an important role inducing bovine deep mycosis in Japan.

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Summary

This study was chiefly undertaken to identify a kind of fungi isolated from eight cases among the ten cattle cases which were brought into the Animal Pathological Laboratory of the College of Dairying for pathological investigation. These ten cattle were presumably suspected to be suffering from mycosis. Isolation of fungi from the lesions was carried out by using malt extract agar incubating at 37°C. One species which was predominantly isolated was identified according to the classification of Lendner and Zych et al. In order to examine the pathogenicity of the fungus, the fungal spore suspension and the concentrate of the fungal cultural filtrate were inoculated into mice intravenously and intraperitoneally, respectively. The pathological examination of the lesions from each cattle and mouse was conducted at necropsy and histopathological observation was made. The fungus was identified as *Absidia corymbifera* as a result of morphological and physiological examinations. The fungus was isolated from various organs of the dead or sacrificed mice which were inoculated with the spore suspension intravenously. The pathological changes in each mouse of the both inoculation and injection were observed. In the microscopic observation of the tissue-sections of the omasum and abomasum from one cow among the ten, big

mycelia which had no septa and which were suspected as the mycelia of *Absidia* were observed in these sections. The fungal colonies were detected in the hay, littler, formula feeds and silage at a barn in which a mycosis cattle case had ever ocurred, but the number was not so great as to directly induce mycosis in healthy cattle under normal condition.

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要　　旨

この報告は，1979年中，本学家畜病学教室に搬入された真菌症の疑いのある10頭の牛について，真菌学的・病理学的検索を行った結果の一部である。この牛のうち1頭は，白血病および第3·4胃の深在性真菌症にかかっていることが判明した。他の9頭は真菌症例ではなかったが，各種の器官より真菌が分離され，特に胃より多く分離された。これらの分離菌は，*Absidia* sp., *Mucor pusillus, Rhizopus* sp. で，最も多かったものは，*Absidia* sp. であり，しかも本邦では未発表のものであった。このため前述の1頭の牛の第3胃より分離された菌を，Lendner, Zycha 等の分類法および佐々木の文献を参照し同定を行った結果，*Absidia corymbifera* と判明した。この菌の胞子懸濁液をマウスに静注し，又この菌の培養濃縮無菌濾液を腹腔内に注射し，病原性・毒性を調査した所，前者では強い病原性を示し，各種の病変も認められた。後者の毒性試験にては多少の変化は観察されたが顕著なものではなかった。この菌の分布を調査するため，以前深在性真菌症の発生した農家の飼料・敷わら中の菌数を調査した結果，多いものでは 4.0×10^4/g のものがあった。これらのことより，本邦においても本菌が真菌症の発生に大きな役割をもっているのではないかと推測される。なお病理学的検索結果については他の論文に記載する。

Explanation of Figures

Fig. 1. Giant colonies of the isolated fungus and *Absidia corymbifera* IFO 4010 on malt extract agar plates incubated at 37°C for 3 days.

Fig. 2. Slide culture of the isolated fungus incubated at 37°C for 2 days.

Fig. 3. A columella of the isolated fungus, remaining frill at the base of the collumella.

Fig. 4. A sporangium and sporangiophore of the isolated fungus.

Fig. 5. Sporangiospores of the isolated fungus.

Fig. 6. Microscopic observation of the kidny-section of a mouse injected intravenously with the isolated fungal spores. An arrow mark shows the invaded hypha.

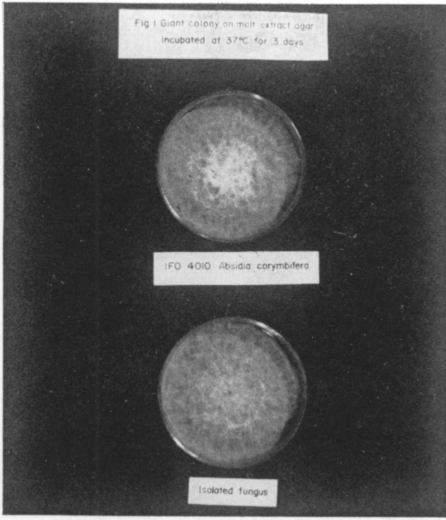

Fig. 1.

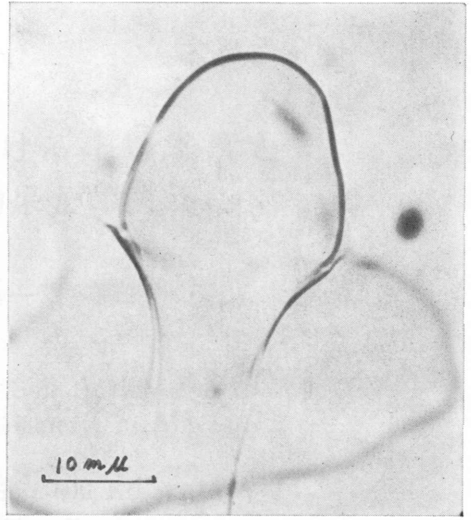

Fig. 4.

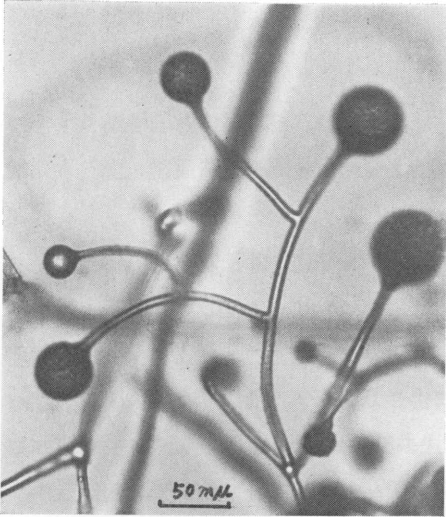

Fig. 2.

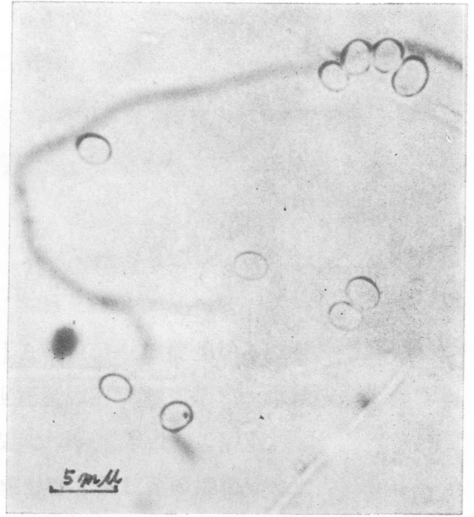

Fig. 5.

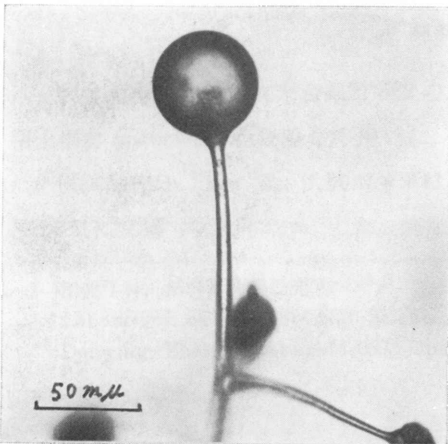

Fig. 3.

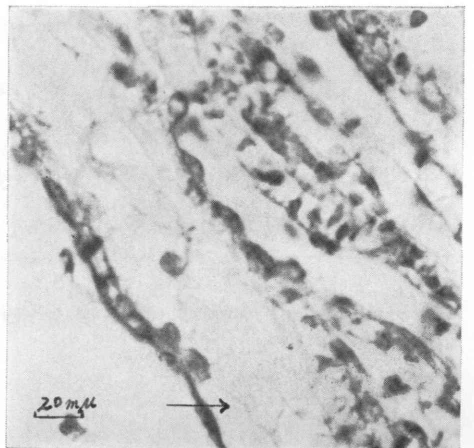

Fig. 6.