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Immune Reaction to Infection by *Malassezia pachydermatis* in Canine External Ear Canals

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개 외이도내 *Malassezia pachydermatis* 감염에 대한 면역 반응

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요 약 : ***Malassezia pachydermatis*균은 개 외이염의 주된 원인균으로 알려져 있으나, 그 감염률은 지역에 따라 많은 차이가 있다. 그러나 그 기병론적인 역할에 대해서는 아직 명확히 밝혀지지 않았다. 이에 본문에서는 국내에서 사육되는 개의 외이염에서 *M pachydermatis*균의 분리율을 조사하고, *M pachydermatis*에 대한 혈중 항체와 외이염과의 연관성을 밝혀 *M pachydermatis*에 의한 외이염 진단을 위한 도구로서 간접 ELISA 법의 유용성을 평가해 보고자 본 연구를 수행하였다. 총 112두 중, 외이염에 감염된 개 44두와 정상견 68두를 대상으로, 심부 외이도에서 취한 가검물을 배양검사하였고, *M pachydermatis* 항원에 대한 혈청내 IgG와 IgM 역가를 간접 ELISA 법에 의해 측정하였다. 살아있는 *M pachydermatis*균을 3두의 정상견 외이도에 접종하여 *M pachydermatis*균의 실험적 감염에 대한 조직병리학적 소견, 조직내 침투 정도, 그리고 접종 후 시간경과에 따른 IgG와 IgM의 혈중 역가변화를 측정하였다. *M pachydermatis*균은 외이염이 있는 개 귀에서 가장 높게 분리(50.0%)되었고, 정상귀에서는 낮게 분리(6.3%)되었다. *M pachydermatis* 항원을 이용한 간접 ELISA 법 진단에서, *M pachydermatis* 양성 배양인 외이염 개체에서 IgG와 IgM에 대한 민감도는 각각 41.7%, 20.8% 이었고 특이도는 88.2%, 85.2% 이었다. 실험적으로 *M pachydermatis*를 접종한 3 두중 1두에서만 조직병리학적 변화와 모낭에 *M pachydermatis* 균체가 확인되었으며, IgG와 IgM 혈중 역가도 유의성 있게 증가하였다. 국내에서 사육되는 개 외이염의 주된 원인균은 *M pachydermatis*균이었고, 이 균의 혈중항체는 유의성 있게 증가하였으며($p<0.005$), 간접 ELISA 법은 *M pachydermatis*에 의한 개외이염의 유용한 진단도구임을 증명하였다.**

Key words : *Malassezia pachydermatis*, IgG, IgM, indirect ELISA, otitis externa

Introduction

Otitis externa of the dogs is a major inflammatory disease of the external ear canal inflicting as much as 20% of the canine population[33,45]. Although it is a common disorder, etiological and predisposing factors and the role of microorganisms in the pathogenesis still remain uncertain. Etiological factors of otitis externa have not been clearly identified and numerous factors are implicated either as primary initiating causes or secondary complications[33,44,45]. Isolation of microorganisms from diseased ears does not necessarily substantiate that these agents are causal, because the ear canal of normal dogs harbors a variety of commensal and potentially pathogenic organisms[37]. When conditions in the ear canal

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are appropriate, these organisms proliferate and initiate or potentiate inflammation and clinical signs of infection.

Predisposing conditions for ear infection include trauma, foreign bodies, *Otodectes cyanosis*, neoplasia, dermatological disorders, allergies, generalized infections, endocrine disorders, and nutritional deficiencies[33,43,45]. In addition, the anatomy of the canine ear predisposes the dog to microbial proliferation that may result in clinical diseases. The external canal is long and narrow and turns medially at the junction of the vertical and horizontal segments. These factors impede clearance of moisture and secretions from the canal. As moisture content increases, the stratum corneum becomes progressively macerated decreasing the protective mechanism and providing a medium for organism proliferation[45].

An organism cultured most commonly from normal and diseased canine ears is the yeast *M pachydermatis*[9,12,21,23,35,37,41]. *M pachydermatis* is distinguished by its ability to grow on complex media without supplementation with fatty acids and no hyphal stage has been described[8,26,38]. *M pachydermatis* has been isolated from dogs, cats, pigs, camels, horses, cattle, elephants and black bears[40]. While *M pachydermatis* is zoophilic[40], the species also has been associated with systemic infections of human and isolated from the humam skin and other clinical specimens such as blood, central venous catheter tips, urine, and tracheal aspirate[2,21,32,34]. *M pachydermatis* has been isolated from clinical specimen of infants and was related to clinical symptoms.

Although *M pachydermatis* is the most commonly associated with canines particulaly as a causative agent of otitis externa in dogs, its pathogenic role is not clearly established. Gustafson[23], who first isolated *M pachydermatis* in canine ear canals, showed that pure cultures of *M pachydermatis* would induce ear infection and isolated the organism from 70% of otitis externa. However, It was pointed out that otitis may be induced experimentally by merely instilling sterile distilled water into the horizontal part of the ear canal. *M pachydermatis* has been frequently isolated in normal canine ear canals, and no significant difference in the number of isolates was found between diseased and normal ears[18]. Later, Fraser *et al*[18] considered that *M pachydermatis* may not be an aetiological agent in otitis externa but that it may be a predisposing factor causing irritation of the lining membrane of the external ear canals.

In more recent studies, *M pachydermatis* was commonly isolated from clinical cases of otitis externa[9,12,19,29,37,41]. For examples, *M pachydermatis* was isolated in 80% of otitic ears and 21% of normal ear[29], and 72% and 36%[9], and 57% and 17%[19], respectively. Especially, Chengappa *et al*[9] proposed that the role of *M pachydermatis* as a possible single cause of canine otitis is worthy of further study, because the presence of *M pachydermatis* was of greater significance statistically in infected ears than in clinically normal ear canals. Mansfield *et al*[28] investigated the role of *M pachydermatis* as a pathogen and the effects of moisture and yeast byproducts in the normal external ear canal of dogs and reported that *M pachydermatis* is an opportunistic pathogen. Despite of a strong association between *M pachydermatis* and otitis externa, isolation rates as low as 8% have been reported from Japan[2], suggesting that geographical differences may be remarkable. There have been, however, no reports against *M pachydermatis* in canine ear canals in Korea.

In histopathological studies on infection by *M pachydermatis*[28], infected ears had hyperkeratosis and acanthosis of the epidermis and a moderate infiltration of lymphocytes into epidermis and dermis. But invasiveness of this yeast and immunological reaction against *M pachydermatis* have not been reported, although *M furfur* has been well established[13,14].

Correlation of antibody response with superficial infection by *M furfur* was well established in human skin disease. Using the indirect immunofluorescent technique, Alexander[1] found antibodies against *P orbiculare* in patients with dandruff. In a report, antibodies against *P orbiculare* were found in both patients with tinea versicolor and controls, but antibody titters were higher in there patients than in controls. In contrast, Faergemann *et al*[16] found no differences in antibody titers against *P orbiculare* between patients with tinea versicolor and adult controls, but observed a significant difference in titers

between adult controls and children. It was thus suggested that although *P orbiculare* is capable of inducing antibodies, these are not correlated to tinea versicolor but occur when an individual becomes colonized with the organisms. But in the following study, Faergemann *et al*[17] found that a significant difference in antibody level between patients in pityrosporum folliculitis and controls. From this result, it was suggested that antibody response may be related to invasiveness of *M furfur*.

It is has been desirable to develope efficient method to diagnosis otitis externa caused by *M pachydermatis* other than culture or direct smear method. Diagnosis of a mycotic infection cannot always be proven by culture or histopathology, despite repeated efforts. Isolation of microorganisms from diseased ears does not necessarily substantiate that these agents are causal, because the ear canals of normal dogs harbor a variety of commensal and potentially pathogenic organisms. When conditions are appropriate, these organisms can proliferate. It is thus necessary to differenciate actual infection from merely proliferation of this organism. In such situation, immunologic procedures can be used to provide rapid and presumptive evidence of infection of *M pachydermatis*.

Immunologic reactions often give the first clues to the current or past fungal infection[24]. Serologic tests can also give information of the effects of chemotherapy and in many cases, lead to increased efforts to isolate and identify the etiologic agent. Antibody responses are thus useful indices in determining the cause and prognosis of a mycosis.

The purpose of the study reported here was to determine the incidence of *M pachydermatis* in clinically normal and otitic dogs in Korea and to evaluate indirect ELISA as diagnostic tools of otitis externa caused by *M pachydermatis* by determining the relevance of antibodies against *M pachydermatis* to otitis externa.

Materials and Methods

Animals and clinical specimens

Dogs under this study included 79 military dogs (Chunchun) and 33 pet dogs(Seoul and Suwon). The age, sex and status of clinical finding were summarized in Table 1. There were 44 dogs with otitic ear(s) and 68 dogs with no apparent ear infection.

Table 1. Studied animals according to age, sex, and presence of skin disease

	Otitic dog			Normal dog		
Age	1-2	3-5	6-9	1-2	3-5	6-9
	18	17	9	30	20	16
Sex	male		female	male		female
	23		22	36		32
Skin	7			3		

Serum samples were obtained by venipuncture. For the culture, sterile cotton applicators moistened by sterile distilled water were used to obtain bacterial and fungal samples. After removing the pinna, the cotton applicator was inserted into external ear canal until reaching horizontal ear canal.

Microscopic examination and culture

Direct smears prepared from ear swab samples were stained write's method and examined microscopically at 1000 x for the presensece of yeasts. The swabs were inoculated onto 5% sheep blood agar and incubated at 37°C for 48 hrs for bacterial cultivation. In order to isolate fungi, the swabs were inoculated onto Sabouraud dextrose agar (containing chloramphenicol 0.05 mg/ml), and the plates were incubated at 37°C, 72 hrs.

Identification of bacteria and yeast

Bacterial colonies were identified based on Cowan & Wteel's tables. Coagulase positive staphylocci were identified using Vitec® GPI card (BioMerieux Vitek. Inc. Hazelwood MO, USA). *M pachydermatis* was identified as described by Slooff[38] and Gordon[20].

Biochemical characteristics were also tested by using Vitec® Yeast card.

M pachydermatis antigen preparation

M pachydermatis cells were cultured in a shaking incubator at 37°C for 3 days in Sabouraud dextrose

broth and centrifuged at 5000 g for 30 min at 4°C. Supernatant was then discarded, and pellets were washed twice with PBS pH 7.2 and resuspended in PBS pH 7.2. The suspension was ultrasonicated at 250W/50 Hz for 60 min with ultrasonicator (Vibra cell, Sonics & Materials. Inc. Danbury, Conn. USA) on melting ice and centrifuged at 5000 g for 30 min at 4°C. The supernatant was collected and centrifuged at 10,000 g for 20 min 4°C and filtered through 0.22 μm pore. The protein concentration of this product was determined using an protein assay kit (Biorad, Richimond, Calif. U.S.A) and used as an antigen in an antibody detection assay.

Detection of antibodies by indirect ELISA

An indirect ELISA was enployed for detection of IgG and IgM antibodies to *M pachyermatis*. The wells of EIA plates (Costa) were coated by incubation with 100 μl antigen solutions (20 μg/ml for protein, in 0.05 M carbonate buffer, pH 9.6) overnigt at 37°C. The coated wells were washed 4 times with PBS with 0.05 % Tween^{20} (PBS-T). The wells were blocked with 5% normal goat serum in PBS-T (200 μl) at 37°C for 1 hr. After blocking solution was discarded, the plates were incubated with 100 μl/well of diluted serum (1:300) in blocking solution. After incubation at 37°C for 1 hr, the plates were washed 4 times in PBS-T. Subsequently, peroxidase-conjugated goat anti-dog IgG or IgM diluted 1:5000 in blocking solution was added and incubated 37°C for 1 hr. The plates were washed again 4 times in PBS-T, and finally, 100 μl of o-phenylenediamine 0.4 mg/ml and H_2O_2 0.04% in citrate buffer pH 5.0 was added. The enzyme reaction was allowed to continue for 15 min and stopped by addition of 100 μl H_2SO_4 (2.5 N). The optical density (OD) at 490 nm was read with an automatic plate reader (Molecular Devices®). The experimental conditions were chosen to give the lowest non-specific background OD reading of normal serum. All samples were tested in duplicate, and an internal standard of pooded serum of dogs with otits externa was run in 4 wells of each plate.

Statistics

The cut-off value for antibody positive samples was determined by adding 3 x s.d. to mean OD value of normal control serum samples. Sensitivity and specificity were determined using this cut-off value and analyzed by Chi-Square test.

Experimental infection of dogs with M pachydermatis

Laboratory animals: 3 healthy mix-breed dogs (1 male and 2 female) were selected from 20 dogs examined by otoscopy. These dogs with normal ears were negative by culture for pathogenic bacteria and fungi including *M pachydermatis*. They were housed in indoor kennels with no contact between dogs throughout this study.

Inoculum: 3 dogs were inoculated only into right ears on days 1, 3, and 5 with *M pachydermatis* prepared from pure culture suspended in 1 ml of physiological saline solution to reach McFarland No. 1 turbidity standard. Left ears were used as control. Inoculum were cultured at the begining to verified viability of the inoculum. Serum samples were collectected on day of 7, 10, 14, 17, 21 later. On day 22, both ears of all dogs were examined otoscopically for evidence of erythema, exudate, or debris, and swabs were taken for culture. The dogs were then euthanized by intravenous barbiturate injection, and the horizental ear canals were excised intact to the tympanic membrane.

Histopathology: Ear canal specimens were fixed in neutral buffered 10% formalin, embeded in paraffin, cut into 3 μm serial sections, and stained with hematoxylin and eosin, Gram's stain, and periodic acid-Schiff (PAS) stain. Each section was examined for the presense of histopathological changes with microscope x.

Results

Cultural findings

Culture results of the dogs under study are summarized in Table 2 and 3. The most frequent isolate from 76 otitic ears was *M pachydermatis* (50%), and followed by *Staphylcoccus aureus*. In normal ears, the most frequent isolate was S aureus, but *M*

Table 2. Microoganisms isolated from otitic and normal ear canals of dogs

Organisms isolated	Infected		Normal	
	No	No/76 (%)	No	No/32 (%)
Malassezia pachydermatis	38	50.0	2	6.3
Staphylococcus aureus	35	46.0	8	25.0
Other bacteria	23	30.7	10	31.3
Fungus	8	10.5	1	3.1
No growth	10	13.2	14	43.8
No. of ear canals	76		32	

Table 3. Frequency of mixed-infection by microorganisms in otitic ears in dogs

Organisms isolated	Infected	
	No	No/66(%)
M. pachydermatis+S. aureus	25	37.9
M. pachydermatis+Other bacteria	5	7.6
M. pachydermatis only	8	12.1
S. aureus+Other bacteria	2	3.0
S. aureus only	8	12.1
Other bacteria+other fungi	6	9.1
Other bacteria only	10	15.2
Other fungi only	2	3.0
No. of ear canals	66	

pachydermatis was rarely cultured. No growth was found more frequently in normal ears (43.8%) than in otitic ear (13.2%). In 8 (21.1%) swabs from the otitic ear canals, only *M pachydermatis* was isolated. In the remaining 30 (78.9%) otitic ear canals, *M pachydermatis* was cocultured with other microorganisms. The presence of *M pachydermatis* was of greater significance in infected ear canals than in clinically normal ear canals ($p<0.005$). The mixed flora of *S aureus* and *M pachydermatis* were found in 37.9% of infected and 3.1% of normal ear canals. The difference between the mixed flora in infected and normal ear canals was statistically significant ($p<0.005$, Chi-spure test).

M pachydermatis was isolated more frequently in female than in male dogs, and the older dogs were, the higher isolation rate was (Fig 1).

When ear swabs smears were stained with

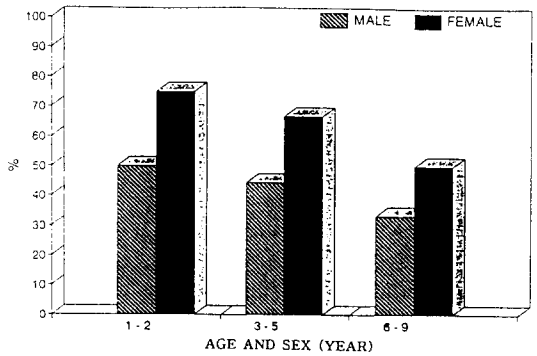

Fig 1. Isolation rate of *M pachydermatis in the otitic ears according to age and sex.*

Table 4. The comparison of direct smear with culture for diagnosis of otitis externa in dogs

Culture	No. of yeast			
	0	1-2	3-10	10<
No growth	26	3	2	
+				
++	1			
+++	1			
TNTC		1	1	1
Total	28	4	3	7
Otitis ear (%)	4(14.2)	2(50)	3(100)	7(100)

Wright's stain and examined microscopically at 1000 x, smears results were closely correlated with culture results (Table 4). Smears from ears with many colonies in culture showed more yeast cells whereas fewer organisms were present on smears prepared from clean ears (Plate 2).

IgG and IgM level to *M pachydermatis*

The results of IgG and IgM level against *M pachydermatis* antigen from otitic and normal controls are summarized in Table 5. ELISA for detection of IgG gave positive result in 10 (41.7%) out of 24 in culture positive otitis cases and 7 (35%) out of 20 in culture negative otitis cases and 8 (12.8%) out of 68 healthy indivisuals. IgM antibodies to *M pachydermatis* was in 5 (20.8%) out of 24, 7 (35%) out of 20 and 10 (14.8%) out of 68, respectively. The sensitivity of IgG ELISA was thus 41.7% in the culture positive otitis group and 35 % in the cul-

Table 5. Indirect ELISA for the detection of circulating antibodies against *M pachydermatis*

Group	Test sera	IgG assay			IgM assay		
		No. of positive	No. of negative	*p* value	No. of positive	No. of negative	*p* value
Culture positive otitic ear	24	10	14	p<0.005	5	19	p<0.1
Culture nigative otitic ear	20	7	13	p<0.01	7	13	p<0.025
Healthy control	68	8	60		10	58	
Cut-off value		0.29			0.15		

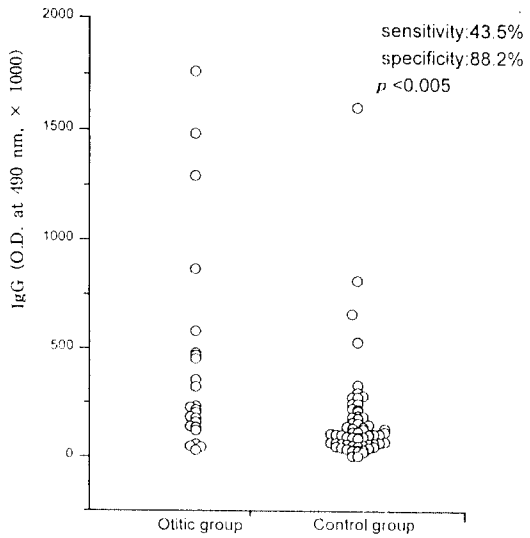

Fig 2. Scatter graph of IgG level from culture positive otitic and normal ears.

ture negative otitis group with a specificity of 88.2%. The sensitivity of IgM ELISA was 20.8%, 35% respectively with a specificity of 85.2%, . *P value* was higher in culture positive otitis group (p<0.005) than any other guoup. Fig 2 shows distribution of IgG level in the culture positive otitis group and control group. As shown in the figure, dogs with otitis externa were more likely to give higher OD than normal dogs.

Table 6. Histopathological finding of canine ear canals experimentally infected with *M pachydermatis*

	Exp. 1		Exp. 2		Exp. 3	
	R	L	R	L	R	L
Clinical score	+	-	+	-	+++	-
Yeast count	++*	-	+	-	++++	-
Invasiveness	-	-	-	-	hair follicle	-
Histopathological findings	-	-	-	-	++++**	-

*Yeast count: ++; ≥ 10/field, +++; ≥ 100, ++++; ≥ 1000

**Hyperkeratosis, acanthosis, and lymphocyte infiltration

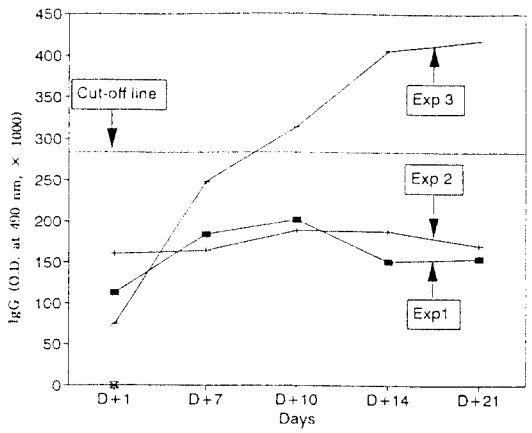

Fig 3. The change of IgG levels in experimentally inoculated dogs.

Infection and antibody response

Table 6 shows the results obtained from dogs infected experimentally with *M pachydermatis*. It was recovered from all 3 dogs on day 22. All 3 infected ear canals appeared in red color, and the lumen contained brown waxy debris. Histopathological changes occured in one animal showing acanthosis and hyperkeratosis of the epidermis and a moderate infiltration of lymphocytes into dermis, and some *M pachydermatis* was found in hair follicles (plates 3-4). Remaining 2 ears were morphologically normal. The untreated left ears were normal by otoscopic

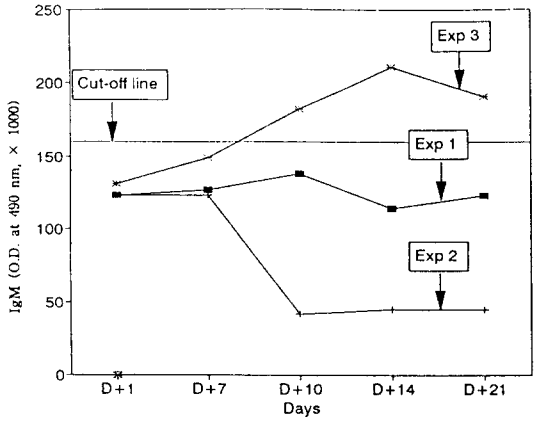

Fig 4. The change of IgM levels in experimentally inoculated dogs.

and microscopical examination and were negative for *M pachydermatis*. Fig 3 and 4 show changes of IgM and IgG levels of the three dogs chronologically from day 1 to day 21. A significant increase in both antibody classes were found only in the dog that had histopathological changes and *M pachydermatis* in its hairfolicles.

Discussion

This study demonstrated that *M pachydermatis* is a major causative agent for otitis externa. *M pachydermatis* was isolated more frequently than any other micro organisms from otitic ear canals. This finding agreed with the results of previous studies[9,12,21,23,35,37,41]. But the isolation rate of *M pachydermatis* was lower from normal ears in comparison with previous reports[9,12,35,37]. The presence of *M pachydermatis* was of greater significance in infected ear canals than in clinically normal ear canals ($p<0.005$). Thus indicating that *M pachydermatis* may be causative agent for otitis externa.

The mixed flora of *S aureus* and *M pachydermatis* was also found in 37.9% of infected and 5.5% of normal ear canals. Frequent association of *M pachydermatis* with *S aureus* was also reported by in the literature[9]. The second most frequent isolate was *S aureus* from otitc ear canals, but from the normal ear canals *S aures* was isolated. Although *S aureus* was the second most frequent isolate from otitic ears, it was also isolated from normal ear canals with the same frequencey indicating no apparent association with otitis externa. Results thus indicated that *M pachydermatis* is the most important microorganism associated in otitis externa of the dogs.

Using the indirect ELISA, we found that circulating IgG and IgM against *M pachydermatis* in greater reactivity in dogs with otitis externa than in controls. Elevated antibodies were found in 41.2% of *M pachydermatis* culture-positive while normal subjects demonstrated lower seroreactivity ($p<0.005$). This result indicates that *M pachydermatis* is capable of inducing antibodies and that the presence of circulating antibodies is correlated to otitis externa.

In experimental infection study, all three ears of inoculated by viable *M pachydermatis* cells showed clinical signs in different levels, but there was only one ear in which a microscopical change occured and *M pachydermatis* cells were seen in the hair follicles. In the dog that had a microscopical change and *M pachydermatis* invasion, antibody reactivity was elevated over cut-off value. This may imply that of the three innoculated dogs, two dogs had no evidence of histopathological changes and yeast cell invasion but an increased in wax and slight erythema of the meatal lining. Despite that many yeasts were cultured in these two dogs, antibody titers did not increased. However, when a high number of yeast cells are present deep in the follicle, antibody production was stimulated.

In the ears of two dogs, despite *M pachydermatis* proliferation and moisture conditions caused by innoculum, no pathologic change was found. Although increased ear wax and slight erythema could be nonspecific response to inoculum than actual infection by *M pachydermatis* cells, the criterion to differenciate normal and otitic ears in this study was the presence of increased ear wax and slight erythema as well as severe symptoms. In culture-positive otitis group, 14 dogs that showed IgG negative might be explained by above reason. *M pachydermatis* is a commensal microorgamism and

Plate 1. *M pachydermatis* cells characterized morphologically as bottle shaped with a unipolar bud attached by a broad base and nonmycelial yeast. Wright's stain, X 1,000.

Plate 2. Direct smear of *M pachydermatis* from canine otitic ear. Wright's stain, X 1,000.

Plate 3. Acanthosis and hyperkeratosis of epidermis caused by *M pachydermatis* infection to canine external ear canal. H & E, X 200.

Plate 4. *M pachydermatis* cells in hairfollicle of canine external ear canal infected with *M pachydermatis*. PAS, X 400.

could proliferate when microenvironment becomes optimal to growth. Proliferation of microoganisms does not necessarily cause disease, even though it could cause disease. In this reason, it may not be reasonable to depend only on culture method for researching infectious agents causing otitis.

Using the indirect immunofluorescence technic, circulating (IgG) antibodies against *P orbiculare* were found in high titers more frequently in patients with *Pityrosporum* folliculitis than in contrlos or in patients with pityriasis versicolor[17]. When yeast cells are present deep in the follicle, antibody production may be better stimulated in comparison with that in tinea versicolor, in which the yeasts are present primarily in the stratum corneum. It was confirmed in this study showing that only the dog with yeast cells in hairfollicles had increased antibodies.

In conclusion, this study shows that *M pachydermatis* is the most important microorganism associated with canine otitis externa in Korea and that indirect ELISA using *M pachydermatis* antigens were useful in diagnosis of otitis externa caused by *M pachydermatis*.

Conclusion

A total of 112 dogs including 44 dogs with otitis externa and 68 dogs with normal ears examined for the presence of antibodies to *M pachydermatis* by indirect ELISA. For experimental infections, three dogs were inoculated into ear canals with *M pachydermatis* and examined for clinical and histopathological changes as well as humoral antibody responses. The results were as follows;

1. *M pachydermatis* was most frequently isolated than any other microorganisms from otitic ear canals, and the presence of *M pachydermatis* was of greater significance in infected ear canals than in clinically normal ear canals.

2. Superficial infections were capable of inducing a humoral responses, and circulating IgG and IgM antibodies against *M pachydermatis* were significantly higher in dogs with otitis externa than in normal dogs.

3. Actual infections with the organism rather than simple colonization is required for production of the elevated antibody levels.

4. Indirect ELISA can be a useful diagnostic tool in detecting *M pachydermatis* as causative agent of otitis externa and monitoring the process of otitis externa

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