A Thesis

For the degree of Master of Veterinary Medicine

Development of Quick Real-time PCR Diagnostic Method for Canine Infectious Dermatitis using GeneChecker

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

Department of Veterinary Medicine

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(Supervised by Professor YoungMin Yun)

A Thesis submitted in partial fulfillment of the requirement for the degree of Master of Veterinary Medicine

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This thesis has been examined and approved.

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Abstracts

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Canine dermatitis is one of the most important diseases, affecting about 10% of dogs. Infectious dermatitis in dogs is caused by a primary or secondary infection with bacteria, fungi, including yeast, and ectoparasites. Diagnosis of this infectious dermatitis consists of physical examination, clinical symptoms, microscopic examination of samples from the lesion site, culture and identification examination, and molecular biological diagnosis. This study was performed to develop a quick real-time PCR kit using a chip for the four main pathogens of infectious dermatitis (*Staphylococcus pseudintermedius*, *Microsporum canis*, Malassezia sp., and *Demodex canis*). Skin and hair samples from canine dermatitis patients who visited the Veterinary Medical Teaching Hospital at Jeju National University were collected and pathogens were identified through microscopic examination, Diff-Quik staining, and culture isolation. As a result of comparing conventional PCR and quick real-time PCR tests using *nuc*, *dp5*, *nad4* and *chs* gene-specific primers for *S. pseudintermedius*, *M. canis*, *Malassezia* sp. and *D. canis*, respectively. It was possible to detect the causative pathogen being detected without cross-reacting with other pathogens. In

particular, the quick real-time PCR test was more convenient and quicker than the conventional PCR. In this study, through the development of the chip-based quick real-time PCR diagnostic method, it is thought that will be a useful point-of-care test(POCT) that can be applied to easily and quickly diagnose the causative bacteria of skin diseases in general veterinary hospitals, not laboratories, and to check the response to treatment.

Keywords: *Staphylococcus pseudintermedius*, *Microsporum Canis,* *Malassezia pachydermatitis, Demodex canis,* Quick real-time PCR

Contents

Introduction

Canine dermatitis is one of the commonest diseases in veterinary hospitals. Dermatitis affects about 10% of the dog population [1]. The causes of canine dermatitis can usually be divided into infectious and non-infectious. Infectious dermatitis is caused by pathogenic organisms in the skin, such as bacteria, fungi, ectoparasites, viruses, etc. In non-infectious dermatitis, there are endocrine, allergic, immune-mediated, and neoplastic causes. Pathogenic organisms in dogs most commonly include bacteria(*Staphylococcus pseudintermedius*), yeasts(*Malassezia pachydermatitis*), and ectoparasites(*Demodex canis*), which are normal inhabitants of the skin.

Clinical signs of canine infectious dermatitis include alopecia, scale, crust, pruritic, papules, and erythematous. Primary skin lesions are characterized in the majority of dogs with dermatitis by intensely pruritic, erythematous, macules, and patches, with the front and hind paws and inguinal regions being the most commonly affected areas. The only clinical signs of infectious dermatitis cannot be used to determine the cause. The clinical signs differ slightly depending on the cause of the infection. There are several tests to diagnose dermatitis. Usually, skin diseases are diagnosed by history taking, physical examination, complete blood count(CBC), serum chemistry, hormone assay, skin scraping, impressive smear and Diff-Quik staining, bacterial culture and sensitivity test, biopsy and histopathological examination, allergy test, hypoallergenic diet test, provocation test, and molecular diagnostic test, etc.

The polymerase chain reaction(PCR) method, which directly detects the genes of infected microorganisms, is the fastest and most accurate method. In the PCR diagnostic test for *Staphylococcus pseudintermedius*, the phosphotransactylase(*pta*), Elonation factor Tu(*tuf*), heat shock protein60(*hsp60*), 16S-rRNA and superoxide dismutase(*sod*)A genes were used in PCR and PCR-RFLP analysis [2, 3]. A real-time PCR using the nucleus chromosome(*nuc*) gene was used for the detection of the *Staphylococcus pseudintermedius* species [3]. Real-time PCR analysis for

diagnosis of *Malassezia* species was identified using specific primers to amplify *Malassezia* 5.8S rRNA, internal transcribed spacer 2(*ITS2*), *ITS3*, and *ITS4* regions [4, 5]. A PCR method for diagnosing *Microsporum canis* using DNA extracted from fungal-infected hair and skin was developed, and there is also a report on the method of discriminating between *M. canis*, *M. audounii,* and T*richophyton* sp. [6]. The chitin synthase(*CHS*) gene was amplified in the PCR diagnosis of *Demodex canis* [7], and the *Demodex canis* real-time PCR method was confirmed with DNA extracted from the roots of hair [8]. DNA extraction from these pathogens and PCR testing are all performed in a laboratory and take approximately 4 hours. However, the new quick real-time PCR is a method that is performed quickly by placing a sample directly into the lysis buffer without DNA extraction and for about 35minutes it on the chip by real-time PCR(CareDX™).

The purpose of this study is to develop a quick real-time PCR diagnostic method for canine dermatitis targeting 4 causal pathogens, which are *Staphylococcus pseudintermedius, Microsporum canis*, *Malassezia* sp. and *Demodex canis*.

Material and Method

Patient information

The patient's samples were taken from 4 patients who visited Veterinary Teaching Hospital of Jeju National University for skin diseases. The dermatitis patients were diagnosed as pyoderma, otitis externa, dermatophytosis and demodicosis. All samples from patient were collected with consent of the guardian(Table1).

Table 1. Information of patients who visited Veterinary Teaching Hospital of Jeju National University for dermatitis

No.	Patient information				Diagnosis	Identified pathogens
	ID	Breed	Age	Gender		
1	A	Welshi gorgi	5	M	Pyoderma	*Staphylococcus* sp.
4	D	Mix	3	F	Otitis externa	*Staphylococcus* sp. *Malassezia* sp.
5	G	Mix	4	F	Dermatophytosis	*Microsporum* sp.
6	H	Mix	5	M	Demodicosis	*Demodex* sp.

Sample collection

All samples were collected from patients who visited to the Veterinary Medical Teaching Hospital of Jeju National University. The skin samples of the lesion area were scraped with sterile cotton swab, and the hair of the lesion were pulled out with forceps, put into a sterilize Eppendorf tube, and kept refrigerated until pathogen identification and examination.

Microscopic examination

For the microscopic examination of bacteria and yeast, a cotton swab of the lesion site was rolled onto a clean slide glass and smeared, fixed with nethanol, and then stained with Diff-Quik staining solution. For the ectoparasitic examination, samples from skin scraping were mounted on the clean slide glass with 20% KOH. Then the slides were examined microscopically for pathogens(OLYMPUS CX31, Japan).

Culture and identification of samples

For bacterial culture, samples taken from skin lesions were inoculated on Luria Bertani(LB) agar and blood agar plate, and then incubated at 37°C overnight. For fungal culture, hairs plucked from skin lesions were inoculated with Sabouraud's dextrose agar(SDA; Difco, MD, USA) and Dermatophyte test medium(DTM, MYKODERMOASSAY TRIO, MEDIVET, KOREA), and then incubated at 25°C for 2 weeks. The morphology of the bacterial and fungal colonies grown on the medium was investigated and microscopic examination was performed.

The Composition of Blood agar (BA) per liter is agar(15.0g), beef extract(10.0g), peptone(10.0g), sodium chloride(NaCl, 5.0g), defibrinated sheep blood(50ml), pH 7.3±0.2 at 25°C and deionized water is added up to 1,000ml. After autoclaving at 121°C for 15 minutes, it is cooled to 45°-50°C, and 15ml of the medium was poured into 90mm diameter sterile plastic petri dishes(Bibby sterilin) and cooled. The composition of Sabouraud dextrose agar(SDA)(Oxoid CM41) per liter is mycological peptone(10g), D-glucose(40g), agar No.1(15g), cyclohexamide(0.4g), and chloramphenicol(0.05g)(Dermasel selective supplement, Oxoid SR075E). After autoclaving at 121°C for 15 minutes and cooling to 50°C, 15mL of the medium was poured into 90 mm diameter sterile plastic petri dishes(Bibby Sterilin) and cooled. The prepared plates were kept in a refrigerator at 4°C and used within 7 days. The

composition of Dermatophyte test medium agar(DTM) per liter is papaic digest of Soybean Meal(10g), dexrose(10g), cycloheximide(0.5g), phenol red(0.2g), chloramphenicol(0.05g), agar(20g) and distilled water is added up to 1,000ml. After autoclaving at 121°C for 10 minutes and cooling to 45-50°C, 15ml of the medium was poured into 90mm diameter sterile plastic petri dishes(Bibby sterilin) and cooled. For the fungal culture, inoculated plates were incubated aerobically at room temperature(15-30°C) for about 14 days.

Standard pathogenic strain

The standard strains used in this experiment are S*taphylococccus pseudintermedius* (ATCC49051), *Staphylococcus intermedius* (KCTC3344), *Staphylococcus delphini* (KCTC3592), *Staphylococcus epidermidis* (KCTC13170), *Malassezia pachydermatitis* (KCTC27588), *Malassezia furfur* (KCTC7743), *Malassezia restrica* (KCTC27527), *Malassezia globose* (KCTC27539), *Microsporum canis* (ATCC11621), *Trichophyton mentagrophytes* (KCTC6316) and *Trichophyton rubrum* (KCTC6375). Except for *Microsporum canis*, all the strains were purchased from the Korean Culture Type Collection (KCTC).

DNA extraction

DNA was extracted from 11 standard strains and pathogens isolated from patients' skin using the QIAamp Mini Kit(Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration (100ng/ul) was finally measured using a spectrophotometer(Nanovue GE, Germany).

Polymerase chain reaction(PCR)

For the identification of the bacteria (*S. pseudintermedius*), yeast (*M. pachydermatitis* and *M. furfur*), fungi (*M. canis, T. mentagrophtes*, *T. rubrum*), and ectoparasite (*D. canis*), PCR was performed using the targeting gene specific primer sets, the nucleus chromosome(*nuc*), the cell death promoting gene(*dp5*), ubiquinone oxidoreductase chain 4(*nad4*) and *chs* gene, respectively (Table 2).

Table 2. Sequences and product size of target gene specific primer sets for PCR

Pathogens	Primer	Sequences	Size	Gene
Staphylococcus pseudintermedius	SP_F SP_R	TGGGCTTGTTCAATCAATATACGA TAATCCTCGTCATTGCCATTGTC	439	*nuc*
Microsporum canis *Trichophyton spp*	MT_F MT_R	TCCACTCYTGGATCGTCAAGC CTTGGAGGAKCCAACGAAGGTAC	420	*dp5*
Malassezia pachydermatitis, *Malassezia furfur*	MP_F MP_R	AATAGAGAACCGATAAGTGTGAATAGGAA CTGATTTCCAAATTGGTATTGATGG	334	*nad4*
Demodex canis	DC_F DC_R	GACCCGGATTATTATGAGTTTGAGG CAACTCTCTCAATTACCTGACTCCATC	304	*chs*

In the reaction mixture for PCR amplification, 100ng of template DNA, 2μl of 10X PCR buffer (2.5mM MgCl, 5mM KCl, 10mM Tris-HCl), 0.5μl of 2.5mM dNTPs (dATP, dGTP, dCTP, dTTP, 2mM each), 0.5U of Taq polymerase (5U/μl Takara, Japan) and 10pmol of primer (10pmol/ul) were included, and the final volume was 20ul with distilled water. PCR was performed with the TAKARA thermal cycler (Takara Biomedicals Co., Japan). The PCR cycle was as follows: initial denaturation at 95°C for 10min, 40 cycles at 95°C for 30sec, 60°C for 30sec, 72°C for 45sec and final cycle 72°C for 45sec. PCR products were loaded on a 1.5% (w/v) agarose (SEA KEM, FMC, USA) gel in TBE buffer (89mM boric acid

and 2mM EDTA, pH 8.0) and electrophoresed at 100V for 20minutes. The PCR products were visualized with a UV transilluminator (Uvitec, UK) and the size of the expected PCR product was confirmed.

Quick real-time PCR

In order to establish the conditions of Quick real-time PCR, each strain purchased from KCTC and DNA extracted from the strain were used. In addition, samples scraped from the skin of a dermatitis patient who came to Veterinary teaching hospital for dermatitis were used. After each sample is put in direct lysis buffer and reacted for 10 minutes(DNA extraction process), 10ul of direct lysis buffer containing DNA is put in dilution buffer and pipetting is performed 5-10 times (DNA dilution process), and each diluted 10ul of the solution was dispensed on a real-time PCR chip(CareDX™) and sealed. For Quick real-time PCR, GeneChecker(GeneSystem, Korea) was used, and the conditions were 40 cycles at 95°C for 300 seconds, 95°C for 30 seconds, 95°C for 5 seconds, and 60°C for 15 seconds. PCR amplification was confirmed graphically in real time.

Results

1. Identification of samples

Diff-Quik staining of the slide glass on which the skin compression smear was applied, and the samples obtained by hair plucking and skin scraping were subjected to microscopic examination by dropping a 20% KOH solution. *S. pseudintermedius* (Figure 1, A) and *Malassezia* sp. (Figure 1, B) was confirmed, and the fungus grown in DTM medium showed *M. canis* spore(Figure 1, C), and adult *Demodex* adult and larva in the sample taken from skin(Figure 1, D) was confirmed.

As a result of applying a skin sample from a dermatitis patient to LB medium, white colonies were cultured, which were smeared on a slide glass and confirmed as *Staphylococcus* sp. by Diff-Quik staining (Figure 2, A). Larger white colonies grew in SDA medium, and peanut-shaped *Malassezia* yeast was confirmed from the results of smearing on slide glass and Diff-Quik Staining (Figure 2, B). As a result of inoculating skin and hair samples in DTM of TRIO medium and culturing, the color of DTM medium changed to red, and spores suspected of *M. canis* could be observed (Figure 2, C).

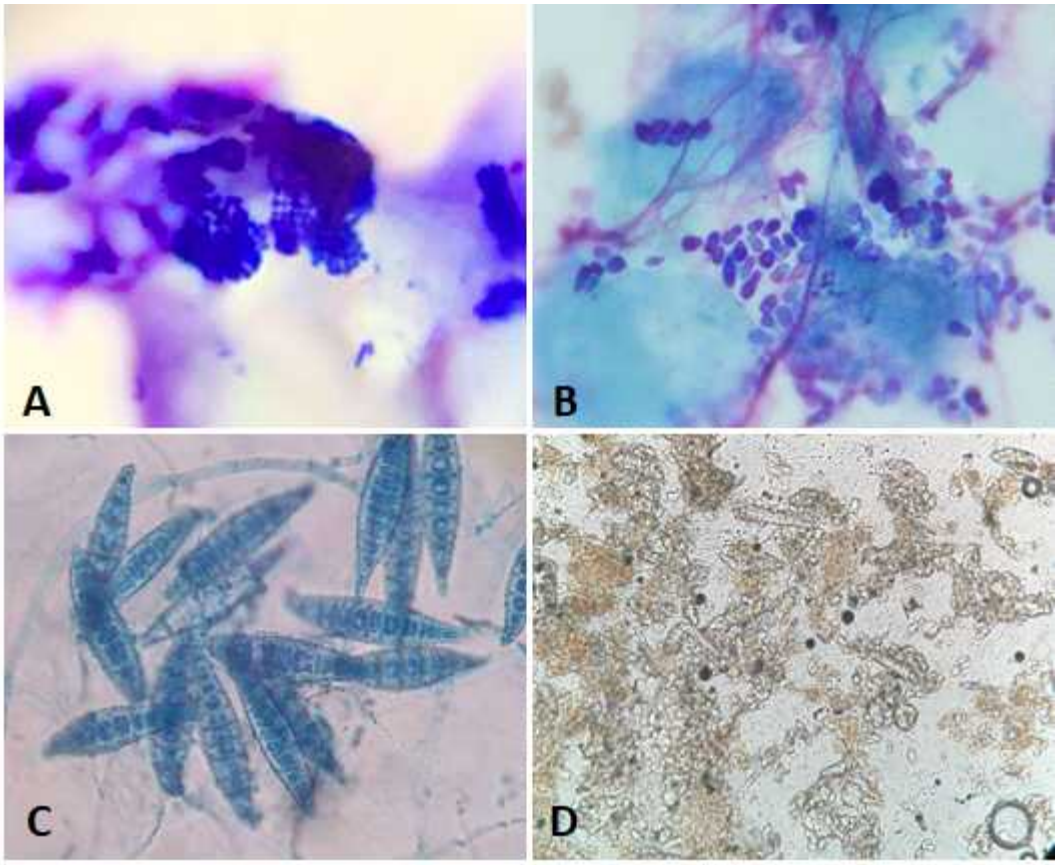

Figure 1. Microscopic pictures of *Staphylococcus pseudintermedius* (A; x1,000), *Malassezia* sp. (B; x1,000), *Microsporum canis* (C; x1,000) and *Demodex canis*(D; x400) (OLYMPUS CX31, Japan).

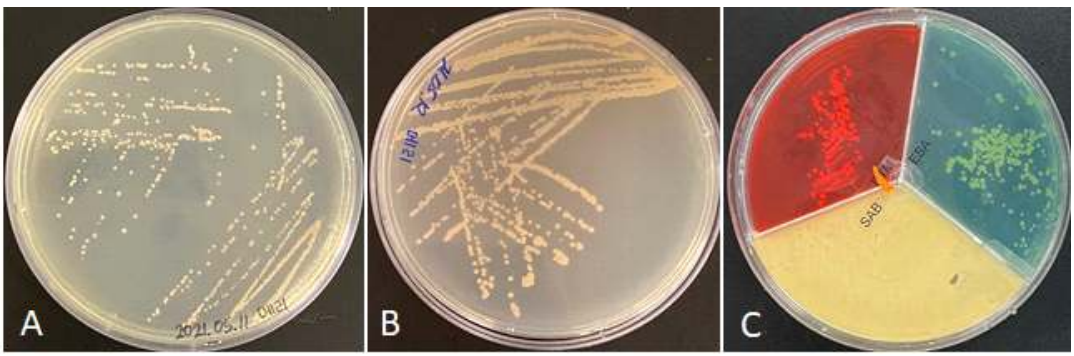

Figure 2. Photographs of culturing the patient's samples are shown in LB(*Staphylococcus pseudintermedius*), SDA(*Malassezia* sp.) and DTM(*Microsporum canis*) media.

2. Conventional PCR

These are the results of conventional PCR analysis using each pathogen-specific primer for bacteria and fungi obtained from the KCTC standard strain and dermatitis patients.

DNA was extracted from the *S. pseudintermedius* standard strain, and the 439 band size expected from the conventional PCR results was shown using a specific nuc gene primer. In addition, PCR amplification was not observed for the nuc gene-specific primers in bacteria other than *S. pseudintermedius*. It was confirmed that only *S. pseudinteremdius* was amplified with the newly prepared nuc-specific primer (Figure 3). Lane 7 is thought to be amplified by a mixed infection of Demodex and *Staphylococcus* sp. as patient sample.

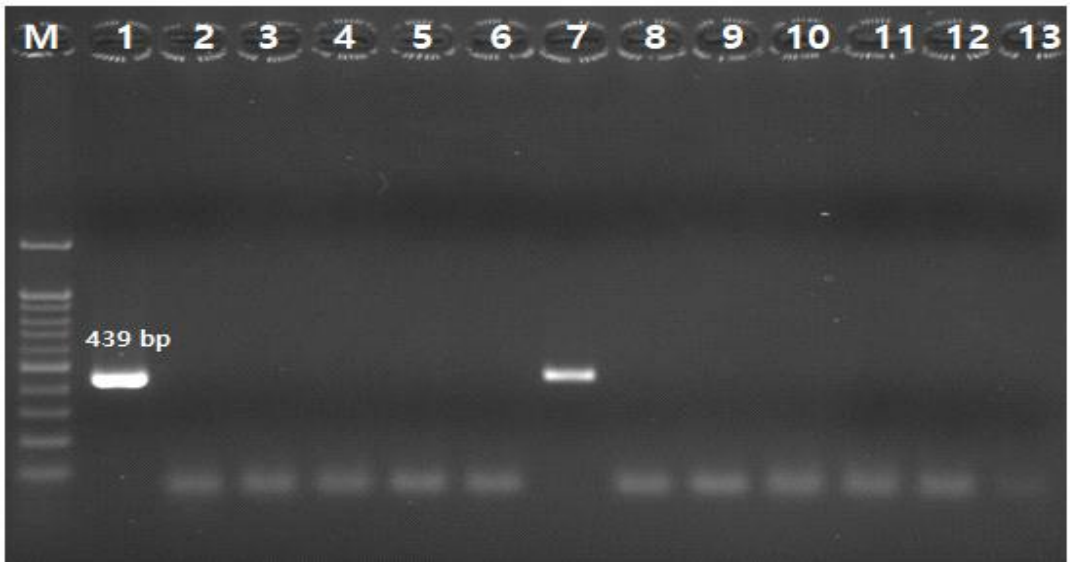

Figure 3. The results of PCR amplification with the *nuc* gene-specific primer set was shown. Lane M indicates 100bp ladders (Bioneer, Korea), Lane 1 *S.pseudintermedius* (ATCC49051), Lane 2 *M.furfur* (KCTC7743), Lane 3 *M.pachydermatitis* (KCTC27588), Lane 4 *M.canis* (ATCC11621), Lane 5 *T.mentagrophytes* (KCTC6316), Lane 6 *T.rubrum* (KCTC6375), Lane 7 *D. canis* (patient sample), Lane 8 S.intermedius (KCTC3344) Lane 9 *S.delphini* (KCTC3592), Lane 10 *S.epidermidis* (KCTC13170), Lane 11 *M.restrica* (KCTC27527), Lane 12 *M.globose* (KCTC27534), Lane 13 NTC (negative control).

DNA was extracted from *M. furfur* and *M. pachydermatitis* standard strains, and a band of size 334 was shown in the conventional PCR results using *nad4* gene-specific primers. In conventional PCR, *nad4* gene-specific primers were amplified only in *Malassezia* sp. without cross-reacting with other bacteria and fungi (Figure 4).

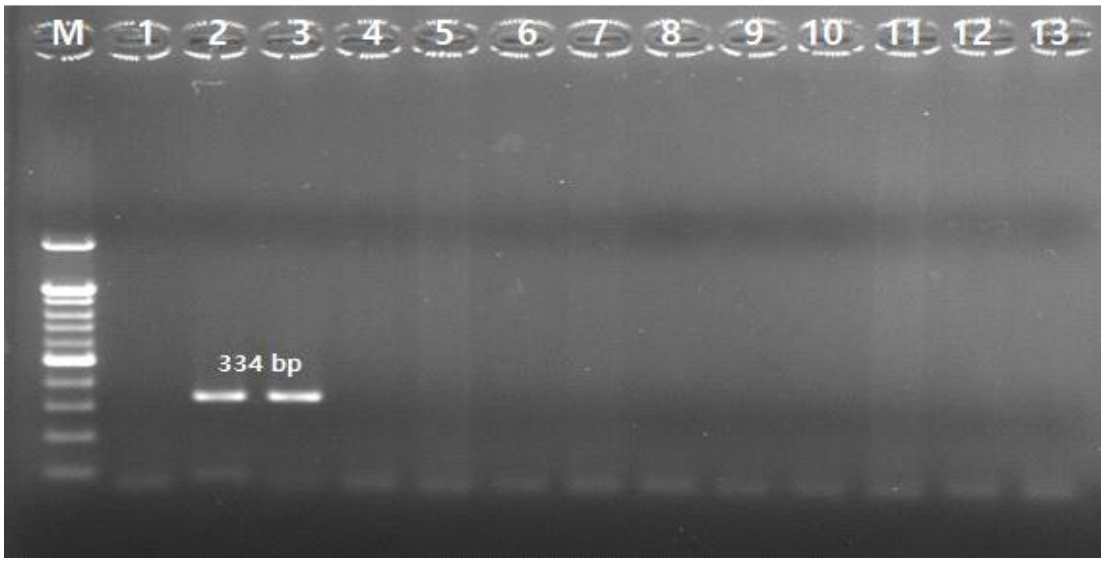

Figure 4. The results of PCR amplification with the *nad4* gene-specific primer set was shown. Lane M indicates 100bp ladders (Bioneer, Korea), Lane 1 *S.pseudintermedius* (ATCC49051), Lane 2 *M.furfur* (KCTC7743), Lane 3 *M. pachydermatitis* (KCTC27588), Lane 4 *M.canis* (ATCC11621), Lane 5 *T. mentagrophytes* (KCTC6316), Lane 6 *T. rubrum* (KCTC6375), Lane 7 *D.canis* (patient sample), Lane 8 S.intermedius (KCTC3344) Lane 9 *S.delphini* (KCTC3592), Lane 10 *S.epidermidis* (KCTC13170), Lane 11 *M.restrica* (KCTC27527), Lane 12 *M.globose* (KCTC27534), Lane 13 NTC (negative control).

DNA was extracted from standard strains of *M. canis*, *T. mentagrophtes*, and *T. rubrum* as microsporum species observed in animals, and conventional PCR was performed with a primer set specific for the *dp5* gene. As a result, only these three fungal strains were shown a band with a size of 420 bp. It was confirmed that the newly prepared dermatophyte *dp5* gene-specific primer was amplified for only three dermatophytes without cross-reaction in other bacteria or fungi (Figure 5).

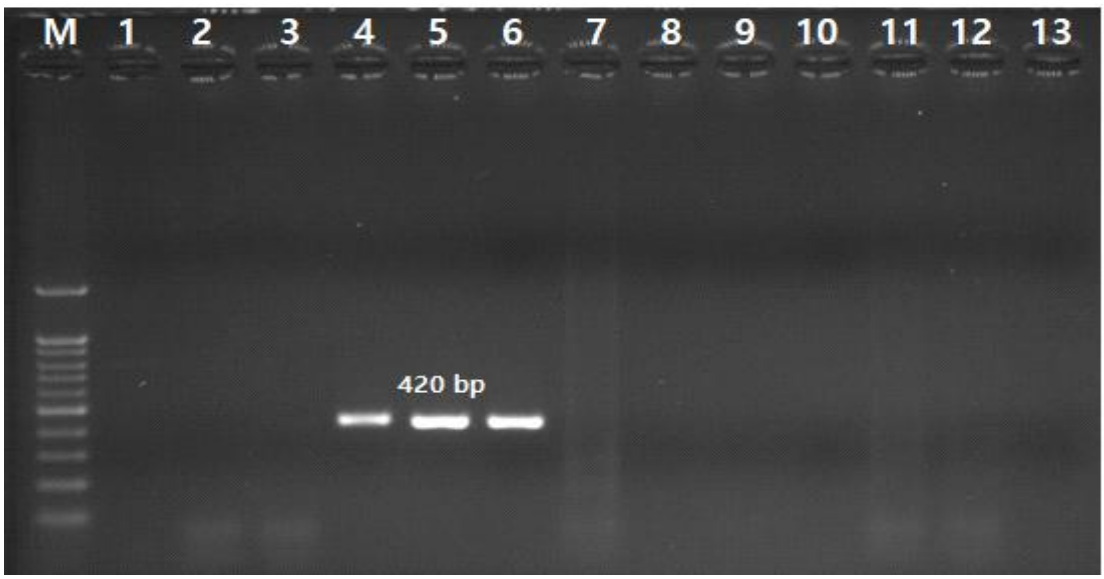

Figure 5. The results of PCR amplification with *dp5* gene-specific primer set was shown. Lane M indicates 100bp ladders (Bioneer, Korea), Lane 1 *S.pseudinteremmedius* (ATCC49051) Lane 2 *M. furfur* (KCTC7743), Lane 3 *M.pachydermatitis* (KCTC27588), Lane 4 *M.canis* (ATCC11621), Lane 5 *T.mentagrophytes* (KCTC 6316), Lane 6 *T.rubrum* (KCTC 6375) Lane 7 *D.canis* (patient sample) Lane 8 *S.intermedius* (KCTC3344), Lane 9 *S.delphini* (KCTC3592), Lane 10 *S.epidermidis* (KCTC13170), Lane 11 *M.restrica* (KCTC27527), Lane 12 *M.globose* (KCTC27534), Lane 13 NTC (negative control).

It was confirmed that Demodex was amplified by PCR amplification using a specific primer set of the Demodex *chs* gene. The chs gene-specific primer did not cross-react with other bacteria, fungi and ectoparasites, and only *D. canis* was amplified. It was confirmed that only the ectoparasite *D. canis* was amplified from demodicosis suspected patient(Figure 6).

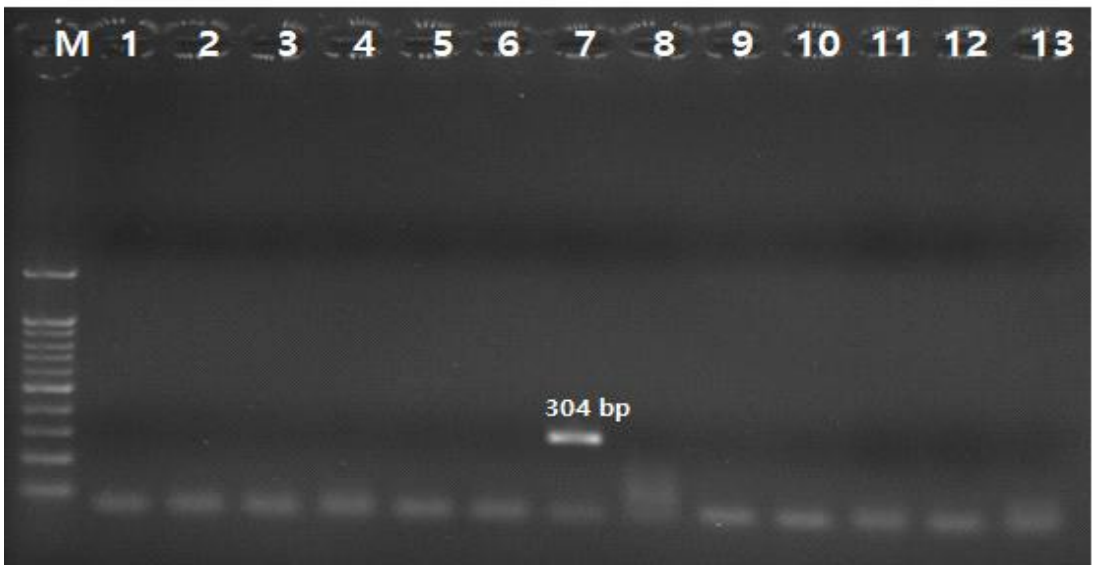

Figure 6. The results of PCR amplification using a *CHS* gene-specific primer set. Lane M indicates 100bp ladders (Bioneer, Korea), Lane 1 S.pseudintermedius Lane 2 *M.furfur* (KCTC7743), Lane 3 *M.pachydermatitis* (KCTC27588), Lane 4 *M.canis* (ATCC11621), Lane 5 *T.mentagrophytes* (KCTC6316), Lane 6 *T.rubrum* (KCTC6375), Lane 7 *D.canis* (patient sample), Lane 8 *S.intermedius* (KCTC3344), Lane 9 *S.delphini* (KCTC3592), Lane 10 *S.epidermidis* (KCTC13170), Lane 11 *M.restrica* (KCTC27527), Lane 12 *M.globosa* (KCTC27534), Lane 13 NTC (negative control).

3. Quick real-time PCR

The results of quick real-time PCR showed results consistent with conventional PCR. The all(*S. pseudintermedius*, *Malassezia* sp, *M. canis* and *D. canis*) pathogens were well detected in quick real-time PCR(CareDX™) based on a chip with a dried specific primer sets. A threshold value(Ct) of 29 suggests a strong positivity, indicating the presence of a lot of nucleic acids of the causative pathogen. A Ct value of 30-37 is positive reactions indicative of moderate amounts of the causative nucleic acid. Ct of 38-40 are considered weak positive with small amounts of the causative nucleic acid that could indicate an infectious state or environmental contamination.

It was confirmed that only *S. pseudintermedius*(ATCC49051) bacteria were amplified from the results of quick real-time PCR using *nuc* gene-specific primer set. The amplified result from the demodex patient sample was thought to have been detected due to a co-infection of Demodex and *S. pseudintermedius*, which was the same as that of conventional PCR(Figure 7).

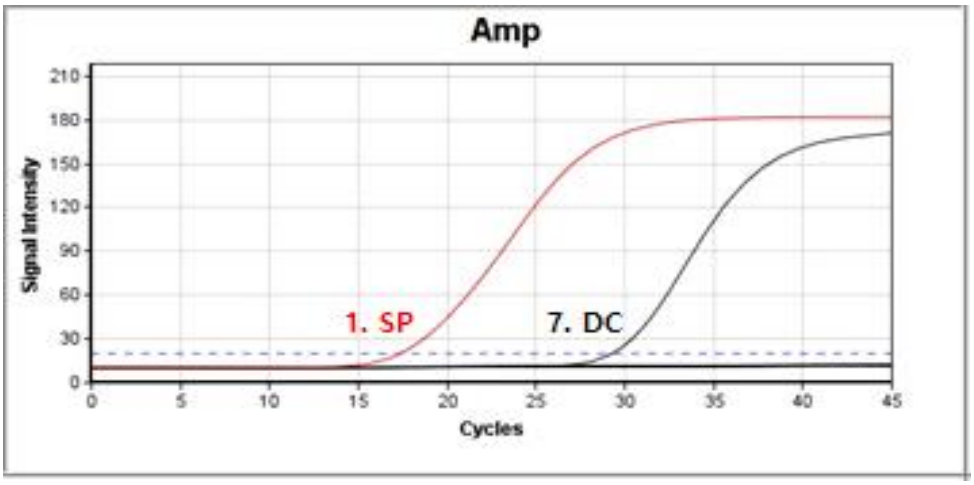

Figure 7. Quick Real-time PCR of *nuc* gene of *S.pseudintermedius* with specific primer sets was amplified shown. Lane 1. SP-*S.pseudintermedius* (ATCC49051), Lane 7.DC-*D.canis* (patient sample). Data not shown from other pathogen by quick real-time PCR.

In quick real-time PCR of *nad4* gene of *Malassezia* sp. with specific primer sets, it was amplified only in standard strains *M. pachydermatitis*(KCTC27588) and *M. furfur* (KCTC7743). It was confirmed that only the yeast *Malassezia* sp. was amplified(Figure 8).

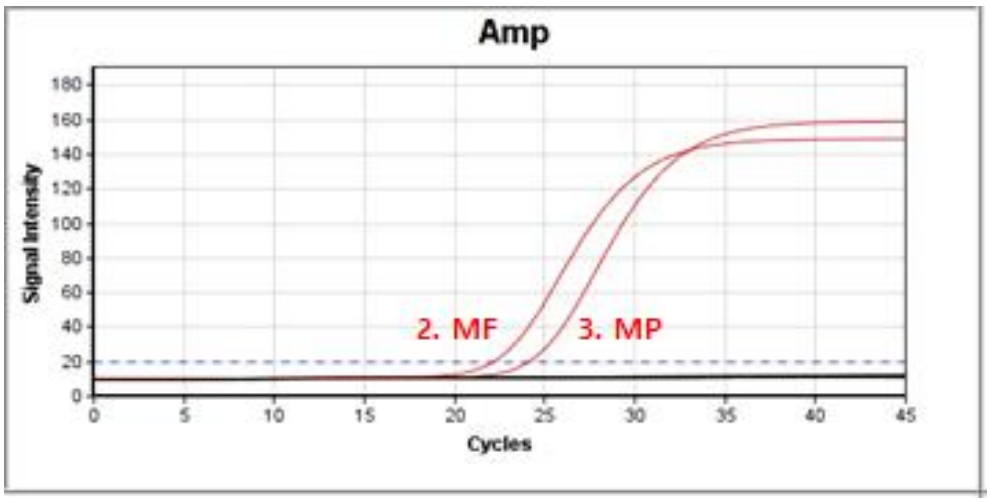

Figure 8. Quick Real-time PCR of *nad4* gene of *Malassezia* sp. with specific primer sets was amplified shown. Lane 2, MF: *M. furfur*(ATCC49051); Lane 3, MP: *M. pachydermatitis.* Data not shown from other pathogen strains by quick real-time PCR.

The result of *M. canis* real-time PCR of *dp5* gene of *M.canis* with specific primer sets, it was confirmed that only the fungi *M. canis, T. mentagrophytes* and *T. rubrum* was amplified from pathogen strain *M. canis* (ATCC11621), *T. mentagrophytes (*KCTC 6316), and *T. rubrum* (KCTC 6375) (Figure 9).

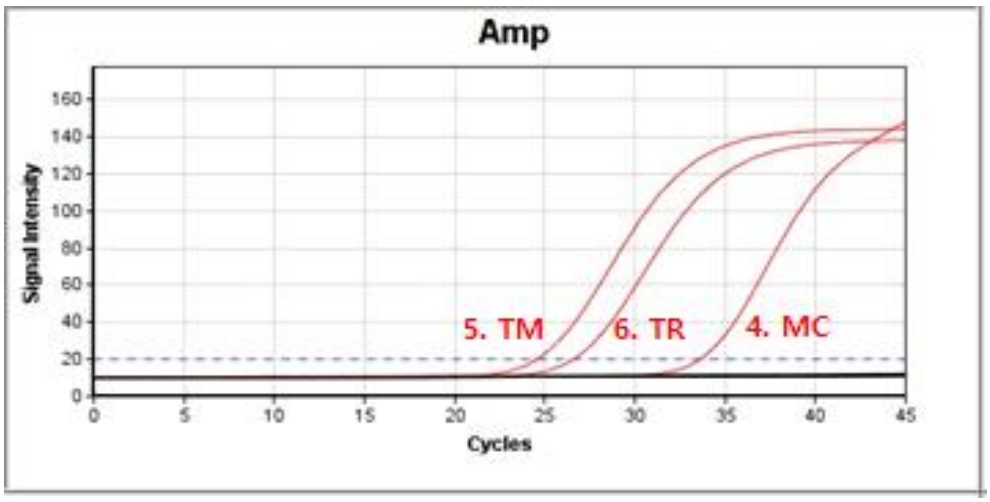

Figure 9. Quick Real-time PCR of *dp5* gene of *M. canis* with specific primer sets was amplified shown. Lane 4, MC: *Microsporum canis;* Lane 5, TM: *T. mentagrophytes* and Lane 6, TR: *T. rubrum*. Data not shown from other pathogen strains by quick real-time PCR.

4. Application of quick real-time PCR in patient

A skin sample of a patient (welshi gorgi, male, 5 years old) caused by *S. pseudintermedius* was evaluated as positive for *S. pseudintermedius* infection by showing Ct of 30 in rapid real-time PCR using *nuc* gene-specific primers. (Figure 10).

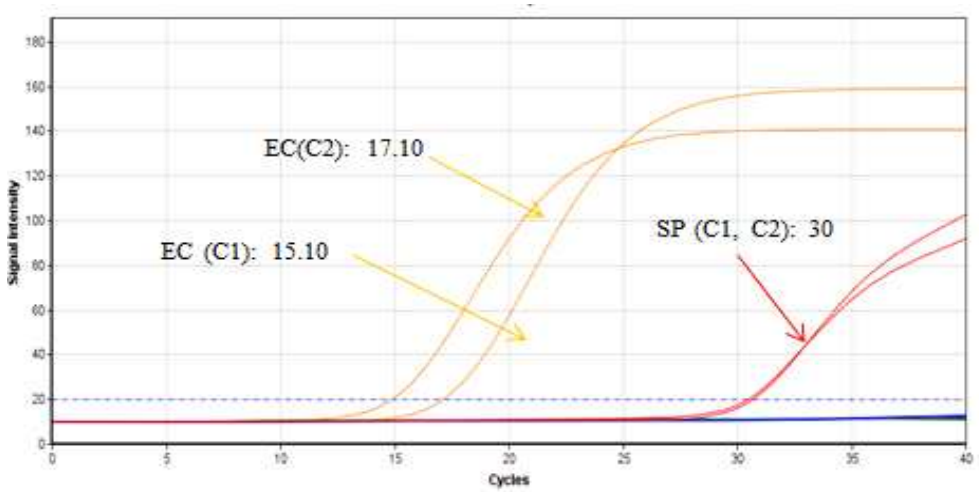

Figure 10. Quick real-time PCR of *nuc* gene of *S. pseudintermedius* with specific primers sets. The yellow lines(EC) are Endogenous control, and the red lines(SP) are *S. pseudintermedius*.

In quick real-time PCR using *nuc* and *nad4* gene-specific primer sets in patient with *S. pseudintermedius* and *Malassezia* sp. co-infection(mix, female, 3 years old), the Ct of *S. pseudintermedius*(SP) was 32.78 and that of *Malassezia* sp.(MA) was 33.40, indicating that two pathogens were mixedly infected. (Figure11).

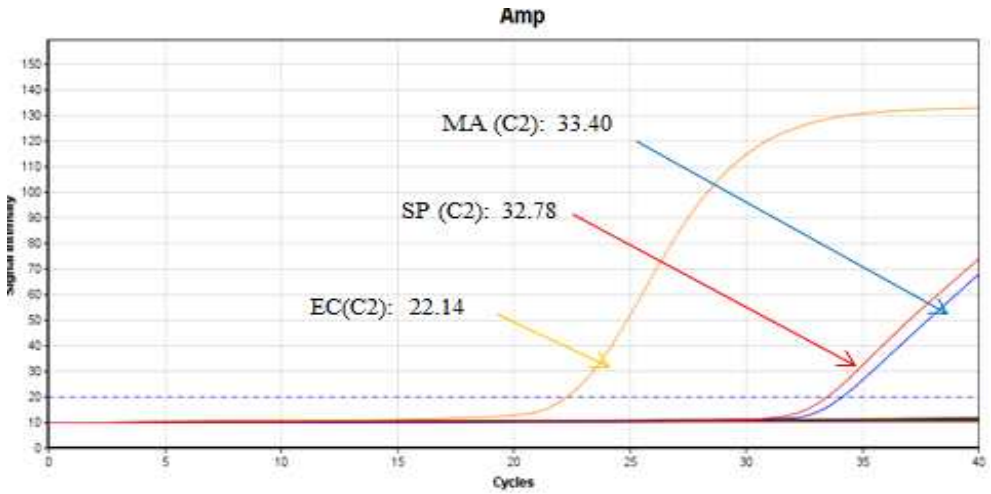

Figure 11. Quick real-time PCR of *nuc* gene of the *S. pseudintermedius*, *nad4* gene of *Malassezia* sp. with specific primer sets. The yellow line is Endogenous control (EC), the red line(SP) is *S. pseudintermedius*, and the purple line(MA) is *Malassezia* sp.

In the quick real-time PCR results using the *dp5* gene-specific primer on the hair and skin samples of a patient suspected of dermatophytosis (mix, female, 4 years old), the Ct value was 30.09, so it was diagnosed as dermatophytosis positive. (Figure 12).

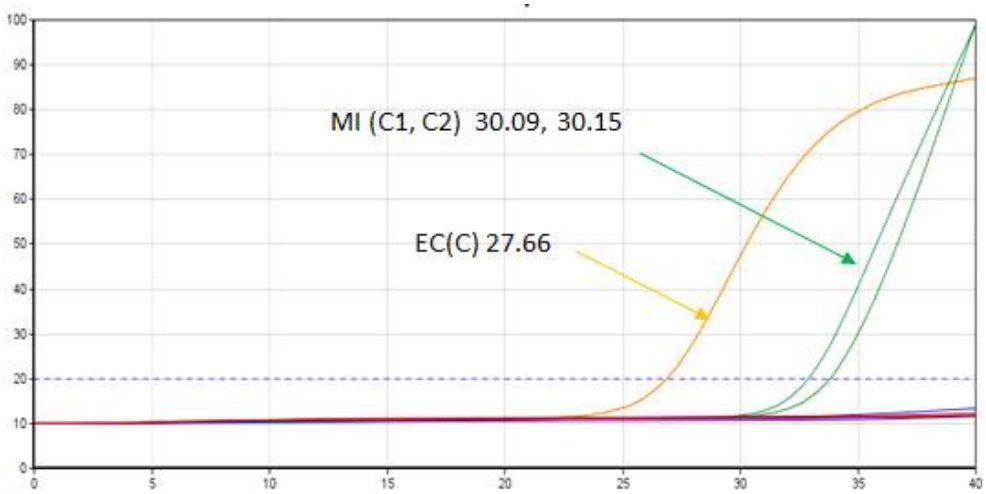

Figure 12. Quick real-time PCR of *dp5* gene of *M. canis* with specific primers sets. The yellow line(EC) is Endogenous control, and the green lines(MI) are *M. canis*.

In the quick real-time PCR results using the *chs* gene-specific primer on the hair and skin samples of a patient suspected of demodicosis (mix, male, 5 years old), It was not amplified by other bacteria, fungi and parasites. It was amplified only with the *chs* gene-specific primer, the Ct value was 30.00, so it was diagnosed as demodicosis positive (Figure 13).

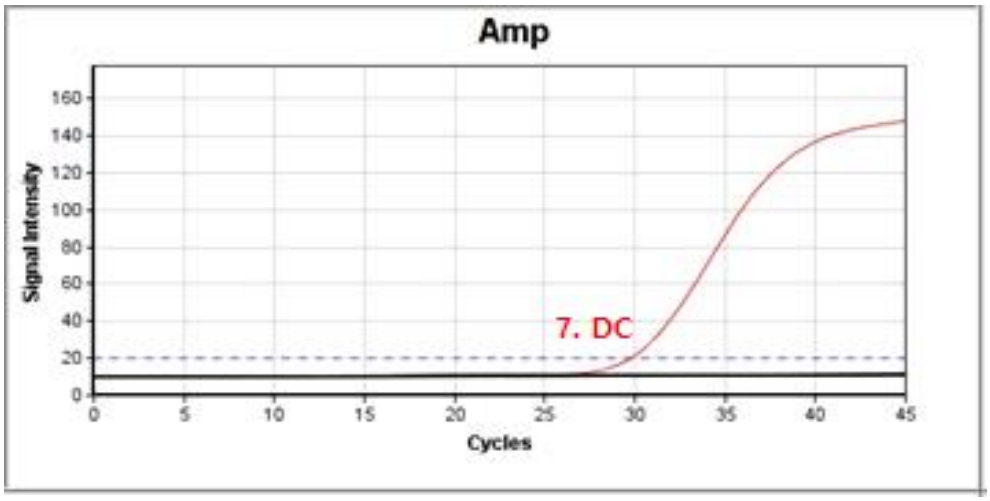

Figure 13. Quick real-time PCR of *chs* gene of *D. canis* with specific primers sets. Lane 7, DC: *D. canis.*

Discussion

S. pseudintermedius, M. pachydermatitis and *D. canis* are normal inhabitants of the skin and mucosa and healthy dogs can be carriers. Canine infectious dermatitis causes pathogen microbiomes. In infectious dermatitis, dog can be colonized by a variety a bacterial, fungal species [9]. It has previously been used in massively parallel sequencing studies. It was found that the predominant bacteria on the skin of dogs are members of Proteobacteria, Actinobacteria and some of the predominant fungi correspond to members of Alternari, Malassezia sp., [10] and some of the predominant ectoparasites are Astigmatina, Psoroptidae [11].

In this study, we've isolated *S. pseudintermedius, Malassezia* sp., *M. canis* and *D. canis* from skin lesion of infectious dermatitis. In normal dogs with healthy skin, *S. pseudintermedius, M. pachydermatitis*, *M.canis* can routinely be isolated by fungal culture, but proving the presence of the organism by skin surface cytology can be difficult [12]. According to previous research, canine dermatitis can be diagnosed almost entirely by microbial culture and cytology [13]. However, these approaches take approximately 3 days to classify bacteria *S. pseudintermedius*, weeks to classify *Malassezia* sp., two weeks to classify *Microsporum sp*. by culture. *D. canis* can be detected directly by cytology. *S. pseudintermedius* was discovered to be the most prevalent bacterium on the skin lesions of dermatitis dogs, while *M. pachydermatitis* was discovered to be more abundant on the skin of dermatitis dogs compared to healthy controls, and bacterial diversity was discovered to be lower at skin sites affected by dermatitis flares in dogs [14]. Also, the fungal marker-gene sequence revealed that the lesion skin of dermatitis dogs had less fungal diversity than healthy skin [15].

Molecular diagnosis was made using two types of conventional PCR and a quick real-time PCR. Most PCR for the detection of the causes of canine skin dermatitis is conventional PCR. The results are apparent only at the end of the reaction and thus, one can't follow the accumulation of the products as it takes place. Quantification of

the products, as in real-time PCR is not possible. However, it has the advantages of being faster and less susceptible to contamination. Our quick real-time PCR method, we can quickly and accurately quantify pathogens *S. pseudintermedius*, *Malassezia sp.*, *M. canis* and *D. canis* in dogs' skin scraping and plucking.

In this study, *nuc* gene PCR(439bp) of *S. pseudintermedius* were successfully amplified from the bacteria samples examined. *S. pseudintermedius* is the most common skin disease in dogs and thought to be the cause of pyoderma [16]. By phenotypic methods, *S. pseudintermedius* can not be clearly distinguished from the other members of the *Staphylococcus intermedius* group (SIG). Recently, a PCR-RFLP test based on a single *Mbol* restriction site in the *pta* gene of *S. pseudintermedius* was developed [3]. It was confirmed that only the bacteria *S. pseudintermedius* was amplified by on chip quick real-time PCR without extraction of DNA. The use of real-time PCR for the identification of *S. pseudintermedius, S. aureus*, and *S. schleiferi* is a valuable tool for achieving better results regarding antibiotic choice and clinical outcome [17].

In this study, isolates of *Malassezia* sp. *nad4* gene PCR product 334bp were successfully amplified from the fungal strains examined. *M. pachydermatitis* can overgrow and act as an opportunistic pathogen in dogs with atopic dermatitis, intertrigo, endocrinopathies, and primary keratinization defects, causing dermatitis and otitis externa. *Malassezia* species have been defined by laborious and complex morphological, biochemical, and physiological characteristics. The 26S ribosomal DNA sequence for *M. furfur, M. pachydermatitis*, *M. obtusa, M. globosa*, *M. restrica*, and *M. slooffiae* was previously reported. The PCR-RFLP method was confirmed to be a simple and effective method using *CfoI* and *BtsCl* [18].

It was confirmed that only the yeast *Malassezia* sp. was amplified by on chip quick real-time PCR without the need for DNA extraction. Quick real-time PCR denotes the quantification of the target gene detected, which includes both living and dead bacteria and yeasts. A responsive, precise, and rapid method for detecting and quantifying skin microbiota from dog skin scrapings. An accurate quantification of *M.*

pachydermatitis yeasts in swab samples from dogs can be achieved with the qPCR method [9].

The *dp5* gene PCR of *M. canis* was successfully amplified from the fungal strains examined in this study. The most often isolated dermatophyte was *M. canis*, which is the main infectious agent of human and animal ringworms. Although several PCR tests for dermatophytes have been described in the scientific literature, only a few are commercially available [19]. described a method for detecting *Epidermophyton* spp. that distinguishes them from *M. canis* or *M. audouinii* without identifying the species [20]. Molecular methods for the detection and identification of *Dermatophytes* from clinical samples have been established in previous studies. The use of PCR in the diagnosis of *Dermatophytosis* has been extensively identified.

It was confirmed that only the fungi *M. canis* was amplified by on chip quick real-time PCR without extraction of DNA. In comparison to traditional methods and conventional PCR methods, real-time PCR assays are well suited to batch processing and therefore have the potential to significantly minimize the amount of time spent manually diagnosing dermatophytosis in the routine clinical laboratory [21].

In this study isolates of ectoparasite *D. canis chs* gene PCR product 304bp was successfully amplified from ectoparasite sample examined. Canine demodicosis is a parasitic infection of the skin in dogs. *D. canis* mites of the same species, taken from different locations, could differ morphologically. The PCR amplification and DNA sequencing confirmed that the mite was a separate species from [22].

It was confirmed that only the ectoparasite *D. canis* was amplified by on chip quick real-time PCR without extraction of DNA. The previous study formally demonstrated, by means of a real-time PCR technique, that demodex mites, albeit in very low numbers, are normal inhabitants of the hairy skin of healthy dogs. It has also been reported that a highly sensitive real-time PCR can detect Demodex at a higher percentage. Quick real-time PCR was able to detect mites in the skin of dogs. The median parasitic load is significantly higher in dogs with generalized and localized demodicosis than in healthy controls [23].

We present here the development of a quick real-time PCR diagnostic method for the identification of *S. pseudintermedius*, *Microsporum canis*, *Malassezia* sp. and *Demodex canis* which achieved specificity. The results of cytologic, culture, and conventional PCR showed the same results as those of quick real-time PCR. Previously, there were many researches to diagnose canine dermatitis with conventional PCR and quick real-time PCR. However, there has been no quick real-time PCR method based on a chip. For definitive diagnosis of canine dermatitis, both quick real-time PCR and conventional PCR tests should be performed concurrently, Although high sensitivity and specific PCR require significant equipment and time for DNA extraction, gene amplification and confirmation. In the future, quick real-time PCR is expected to become an easy, rapid and effective point-of-care test(POCT) tool for canine dermatitis.

Conclusion

This study was performed to develop a quick real-time PCR kit using a chip for the four main pathogens of infectious dermatitis(*Staphylococcus pseudintermedius*, *Microsporum canis*, *Malassezia* sp., and *Demodex canis*).

Skin and hair samples from canine dermatitis patients who visited the Veterinary Medical Teaching Hospital Jeju National University were collected and pathogens were identified through microscopic examination, Diff-Quik staining, and culture isolation. As a result of comparing conventional PCR and quick real-time PCR tests using *nuc*, *dp5*, *nad4* and *chs* gene-specific primers for *S. pseudintermedius*, *M. canis*, *Malassezia* sp. and *D. canis*, respectively. It was possible to detect the causative pathogen to be detected without cross-reacting with other pathogens. In particular, the quick real-time PCR test was more convenient and quicker than the conventional PCR.

Through the development of the quick real-time PCR kit in this study, it is thought that will be a useful diagnostic method that can be applied to easily and quickly diagnose the causative bacteria of skin diseases in general veterinary hospitals, not laboratories, and to check the response to treatment.

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국문초록

Korean abstract

GeneChecker를 이용한 개 감염성 피부염 진단을 위한 신속 실시간 PCR 진단방법의 개발

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개 피부염은 개의 약 10 %에서 발생하는 가장 중요한 질병 중의 하나이다. 개 감염성 피부염은 세균, 효모를 포함한 곰팡이 그리고 외부기생충의 1차 또는 2차 감염으로 인해서 발생합니다. 이들 감염성 피부염의 진단은 신체검사, 임상증상, 병변부위의 시료를 통한 현미경 검사, 배양 및 동정검사 그리고 분자생물학적 진단으로 이루어집니다. 본 연구에서는 감염성 피부염의 주된 4가지 병원체(*Staphylococcus pseudintermedius*, *Microsporum canis*, *Malassezia* sp., *Demodex canis*)에 대해서 칩을 이용한 신속 실시간 PCR 진단 방법을 개발하고자 수행하였습니다. 제주대학교 수의과대학 부설동물병원에 내원한 개 피부염 환자의 피부와 털 시료를 채취하여 현미경 검사, 염색 및 배양 분리 동정을 통해서 병원균을 확인하였다. 포도상 구균(*S. pseudintermedius*), 피부사상균(*M. canis*) 효모균인 말라세치아(*Malassezia* sp.) 그리고 모낭충(*D. canis*)에 대한 각각 *nuc*, *dp5*, *nad4* 및 *chs* 유전자 특이 프라이머를 통해서 일반 PCR 및 신속 실시간 PCR 검사를 비교한 결과, 2가지 모두에서 다른 세균, 곰팡이와 기생충과의 교차 반응없이 검출하고자 하는 원인 병원체를 검출할 수 있었다. 특히 신속 실시간 PCR 검사는 기존의 일반 PCR에 비해서 좀 더 간편하고 빠르게 결과를 확인할 수 있었다. 본 연구의

신속 실시간 PCR 진단방법을 통해서 실험실이 아닌 일반 동물병원에서도 간편하고 빠르게 피부병의 원인균을 진단할 수 있고, 치료에 대한 반응을 확인하는 데에도 적용할 수 있는 유용한 현장 진단법(POCT)이 될 것으로 생각된다.

중심어: 포도상구균, 효모균, 피부사상균, 모낭충, 신속 실시간 PCR

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처음 지도교수님으로부터 피부병을 주제로 논문쓰라고 했을 때, 무엇부터 해야할지 모르고 걱정이 많았습니다. 케어벳(CareVet) 문명진 박사님의 도움으로 세균, 곰팡이와 기생충 유전자 증폭을 위한 프라이머 제작 그리고 일반 PCR과 신속 실시간 PCR 실험을 잘 할 수 있도록 도와주셔서 지금의 논문을 쓸 수 있었습니다. 논문 데이터를 잘 분석하고 만들 수 있도록 도와주신 문명진 대표님과 최지영 팀장님께 감사드립니다.

모기와 진드기 채집을 함께 했던 한라대학교 김지로 교수님과 문혁, 김원근, 공현우, 이홍석과의 즐거웠던 추억들을 오래 간직하겠습니다. 그리고 반려동물 내과학 실험실의 허은지 선생님은 매일 아침 옆자리에서 웃으면서 저를 맞아주셨고, 동물병원 진단검사와 연구 실험 그리고 제 학위논문을 쓰는데 많은 도움을 주셔서 감사합니다. 자주 뵙지는 못했지만 제주야생동물구조센터의 진태정 실장님과 장진호 박사님에게도 감사드립니다. 실험을 위해서 연구실에 와서 안부도 물어주시고 생활에 도움을 주신 HRG 장진욱 박사님 그리고 컴퓨터 공학과 곽호연 교수님께도 감사드립니다.

내과학 실험실에서 같이 생활하면서 많은 도움도 주고 잘 생활할 수 있도록 챙겨준 대학원생 김민건, 구지영, 조희수 선생님, 올해(2021년)에 졸업한 윤종필, 엄희성, 백재훈, 이동재, 이승준, 정서영 수의사 그리고 지금 학교 다니고 있는 강정순 언니, 박종진, 조원영, 정윤희, 하현종, 유준석, 김민지, 이도현, 백세영 학부생에게도 감사드립니다. 동물병원의 고인종 팀장님, 이성일 수의사님, 전지현 선생님, 양수연 선생님께도 감사드립니다. 수의대 4층 내과실험실과 부설동물병원에서 연구하고 생활하면서 몽골 고향 생각도 많았지만, 낯선 한국 제주도에서 잘 적응하고 생활할 수 있도록 많은 도움을 주셔서 감사드립니다. 그리고 수의학과 사무실 김보라 조교님과 오보미 조교님께도 감사드립니다. 외국학생인 저에게 학교생활에 필요한 정보와 서류들을 잘 챙겨주셨고 설명도 잘 해주시고, 신경도 많이 써주셔서 다시 한번 감사드립니다.

짧았던 제주에서의 2년을 재미있게 잘 생활할 수 있도록 도와주셨던 앞에서 말하지 않은 모든 분들을 결코 잊을 수 없고, 너무너무 감사하고 제 마음 속 깊이 항상 기억

될 것입니다. 8월에 한국 제주도를 떠나 고향인 몽골로 가지만, 언젠가 한국이나 몽골에서 다시 볼 수 있기를 기대해 봅니다.

Мөн дипломын ажил бичих хугацаанд тусалж дэмжиж урам зориг өг ч бай сан най з Энхбадрал, Зулмандах, Энхтуяа, Дүүрэнжаргал, Билгүү н, нартаа маш их баярлалаа. Их сургуулий н амьдралыг хамтдаа дава н туулж сай хан цаг хугацааг хамт өнгөрүүлсэнд баярлалаа.

Най з Должинсүрэн дээ маш их баярлалаа. Солонгост ирж сурах хүсэл зориг нэмж, тусалж хаана ч дэргэд минь бай гаа юм шиг л зааж зөвл өж ярилцаж ирсэнд баярлалаа.

Мал эмнэлгий н сургуулий н проффесор багш Ням-Осор багшдаа баяр лалаа. Мал эмнэлгий н мэргэжлээр ахин суралцах хүсэл зориг нэмж, тусалж дэмжиж бай санд баярлалаа.

Мөн эгч нар болох Бямбацэцэг, Оюунцэцэг, Мөнхцэцэг, Бадамцэцэг нартаа баярлалаа. Намай г хай рлаж хамгаалж дэмжиж урам зориг өгч бүхий л зүй лээ хуваалцаж, зааж сургаж ирсэнд баярлалаа.

Сүүлд нь намай г бүхий л боломжоороо дэмжин, дүүрэн хай раар ха й рласаар бай сан хай ртай Аав Отгонбаяр, ээж Хишгээ та 2доо маш их баярлалаа. Хэдий хол бай сан ч яг л хажууд минь түшиж бай гаа юм шиг бай сан та хоёрдоо баярлалаа.

2021년 7월 5일, 석사학위 논문 마지막 교정을 하면서

오트곤 오르곤체첵

OTGONBAYAR OTGONTSETSEG