Development of *pmp* Gene-Specific PCR Assay with A Host Specific Internal Control for *Chlamydophila felis*

Zafer Cantekin[1*] Hasan Solmaz[2] Nuri Altug[3] Gamze Ozge Ozmen[1]

Abstract

Chlamydophila felis is the primary bacterial agent of conjunctivitis and upper respiratory disease in cats. Transmission of the disease requires close contact between cats. Polymerase chain reaction is a useful tool for detection of this organism. The aim of this study was to develop a polymerase chain reaction assay with an internal amplification control for the detection of *C. felis*. Primer pairs were designed specifically for *polymorphic membrane protein gene* of *C. felis* and *cytochrome b gene* of cat, and their specificity and sensitivity were examined. Primers specific for both genes were then multiplexed. In the simplex polymerase chain reaction analyses with 10-fold dilutions, *C. felis* DNA was detected with designed primers for *polymorphic membrane protein* genes up to 1.6 pg/µl and cat DNA was demonstrated in all samples in the polymerase chain reaction. Moreover, in the multiplex polymerase chain reaction, *C. felis* DNA and cat DNA were detected together. These designed primers specific for *C. felis* might have potential for research on infections and shedding of this organism in cats as the internal control host specific primers might have potential for using internal control for detection of different microorganisms in cats.

Keywords: Cat, *Chlamydophila felis,* multiplex polymerase chain reaction, *polymorphic membrane protein gene*

[1] *Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Microbiology, Tayfur Sokmen Campus, Hatay 31000, TURKEY*
[2] *Yüzüncü Yıl University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Campus, Van 65100, TURKEY*
[3] *Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Internal Disease, Hatay 31000, TURKEY*
***Correspondence:** zcantekin@hotmail.com*

Introduction

Chlamydophila felis (*C. felis*) is the causative agent of feline chlamydiosis. The agent was described as a feline subtype of *C. psittaci* in the past, but it is now classified in the *Chlamidophyla* genus of the *Chlamidaceae* family (Everett et al., 1999). *C. felis* causes upper respiratory system disease, including conjunctivitis. The disease is transmitted between cats through contact with infected tears. Young cats are more sensitive than older animals to infection, and crowded conditions are also important in the epidemiology of the disease. Shedding of the agent can range from 2 months to 215 days (Wills et al., 1987; Sykes, 2005). Owners of infected cats can also develop conjunctivitis and pneumonia. Infected immunosuppressed individuals can develop glomerulonephritis, endocarditis, and hepatitis (Rodolakis and Mohamad, 2010).

The first step in the diagnosis of feline chlamydiosis is staining of conjunctival smear, but this is not a reliable technique (Streeten and Streeten, 1985). For the isolation of the agent culture in egg yolk and cell culture can be used, but they are time consuming procedures and they need special laboratory and experts. Because of these type disadvantages of conventional methods, Molecular techniques have been used for the detection of the agent. Molecular techniques have been reported to be more sensitive and successful in the diagnosis of the agent (McDonald et al., 1998; Sykes et al., 2001). Moreover, polymerase chain reaction (PCR) was emphasized that this assay should be performed again after therapy due to the extended shedding time of the bacterial agent (Sykes et al., 1999).

A number of studies showed that *polymorphic membrane protein* (*pmp*) gene-specific primers had high specificity in the molecular detection of *C. abortus* and *C. psittaci* (Laroucau et al., 2001; Greco et al., 2005; Laroucau et al., 2007; Sareyyupoglu et al., 2008). Research also reported that polymorphic membrane proteins encoded by *pmp* genes are a useful target for vaccines for *Chlamydia* spp. (Longbottom and Livingstone, 2004). Similarly, the presence and distribution of *pmp* genes and proteins in *C. felis* have been described (Harley et al., 2007), and *pmp1* and *pmp7* genes were shown to be immune-dominant proteins in *C. felis* (Harley et al., 2010); however, there is not any report on using *pmp* genes specific primers in the PCR detection of *C. felis*.

In PCR analyses, negative results may not always reflect reality. To detect false negative results caused by inhibitors or mistakes in the various stages of PCR analysis, use of internal amplification control (IAC) is recommended. The target gene and IAC are amplified together in the same PCR reaction. The inability to amplification of the IAC can reflect failure in the PCR amplification such as dysfunction of the thermal cycler, mistakes in PCR mixture or protocols, failure in polymerase activity, and especially presence of inhibitory substances in the samples (Anonymous, 2002; Hoorfar et al., 2003). There are many strategies for using internal controls. For this purpose, a template as an internal control can be chosen in the clinical material or it can be added to material or PCR mixture exogenously (Hoorfar et al., 2004; He and Shi, 2010).

In this study, we aimed to develop PCR analyses with an internal amplification control to detect *C. felis*. For this purpose, we designed specific primers for *pmp* genes of *C. felis* and then combined these primers with cat-specific primers for *cytochrome b* genes (*cytb*).

Materials and Methods

***Preparation of positive control DNA*:** *C. felis* DNA was used as a positive control. *C. abortus, C. psittaci* (The authors would like to thank Dr. Nicole Borel, Dr. Vladimir Demkin, Dr. Mustapha Berri and Dr. Nieves Ortega for *Chylamydia* DNA) *Escherichia coli*, *Brucella abortus* S19, *B. melitensis* 16M and *Staphylococcus aureus* DNA (The authors would like to thank Dr. Ziya Ilhan) was used for detecting the specificity of the primers. After the detection of the specificity of the primers (Cfpmp1a/Cfpmp1b and Cfpmp1c/Cfpmp1d), 10-fold dilutions of *C. felis* DNA (16 ng/µl) were prepared to determine the sensitivity of these primers. A NanoDrop spectrophotometer (ND 1000, USA) was used to measure the concentration of all DNA in this study.

***Clinical Specimens and DNA isolation*:** In total, 77 swab samples (61 conjunctival and 16 nasal) were obtained from 43 cats with upper respiratory tract disease and conjunctivitis living in four different cat shelters in Hatay, Turkey (The clinical samples were taken with permission with MKÜ Local Ethics Committee, Meeting Date 09.06.2011: Meeting No: 2011/04: Decision No: 7). Clinical signs of cats and properties of samples are shown in Table 1. The swabs were kept in 1 ml of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) for 30 min. DNA was then extracted from this buffer with the ether-chloroform method. The extracted DNA was stored at -20°C until PCR analyses (Sambrook and Russel, 2001).

For multiplex PCR analyses (*C. felis* and internal amplification control with host specific primers), 10-fold dilutions of *C. felis* positive control DNA were added to DNA from conjunctival swab samples negative for *C. felis*. One µl of this mixture was used as a template experiment and subjected to PCR analyses with the internal control.

***Primer Design*:** Original primers specific for the *C. felis pmp1* gene (GenBank accession no.EF092092) and for the internal amplification control primers specific for the mitochondrial *cytb* gene of *Felis catus* (GenBank accession no. AB194813) were designed using Primer3 (http://www.ncbi. nlm.nih.gov/tools/primer-blast/) (Yuryev, 2007). Properties of the primers are shown in Table 2.

***Optimisation PCR procedures and protocols*:** All procedures and protocols for individual PCR and multiplex PCR assays were optimised (Henegariu et al., 1997). The PCR amplification mixture was placed in a final volume of 25 µl. The mixture consisted of 1 µl of DNA template with 10-fold concentration of *C. felis* DNA and different concentrations of clinical samples. In all of the simplex and multiplex PCR analyses, the

PCR mixture consisted of 1 µl of DNA template, 1.5 of TaqDNA polymerase, 3.5 µl of 10× PCR buffer (10X ViBuffer A, without $MgCl_2$), 3 mM of $MgCl_2$, 200 µM each of dNTPs, and 20 pmol of each primer. After initial denaturation at 95°C for 3 min, the PCR protocol was: 60 s of template denaturation at 94°C, 60 s of primer annealing at 54°C, and 90 s of primer extension at 72°C (total of 30 cycles), with a final extension at 72°C for 5 min. The samples were analysed by electrophoresis in a 2% agarose gel and stained with ethidium bromide (0.5 mg/mL), and DNA bands were visualized under UV light.

Results

In the PCR with the Cfpmp1a/Cfpmp1b and Cfpmp1c/Cfpmp1d primers for specifity analyses, specific products were amplified in *C. felis* control DNA, but no amplification products were observed with *C. abortus*, *C. psittaci* or with other control DNA (data not shown). In the PCR analyses with 10-fold dilutions of *C. felis* DNA, the Cfpmp1c/Cfpmp1d primers (1.6 pg/µl) were 10 times more sensitive than the Cfpmp1a/Cfpmp1b primers (16 pg/µl). The

Table 1 Shelter, Gender, Age and Clinical Signs Data for Cats and Origin of Swab Samples

Cat No	Shelter and Cat No	Gender	Age	Conjunctival Swab Left/Right	Nasal Swab	Clinical Signs
1	Shelter1-1	Male	2 Months	+/+	-	Conjunctivitis
2	Shelter1-2	Female	3 Months	+/+	+	Conjunctivitis, Nasal Discharge
3	Shelter1-3	Female	2 Years	-/+	-	Conjunctivitis
4	Shelter1-4	Female	6 Years	+/-	+	Conjunctivitis, Nasal Discharge
5	Shelter1-5	Female	5 Years	+/-	-	Conjunctivitis
6	Shelter1-6	Female	3 Years	+/-	-	Conjunctivitis
7	Shelter1-7	Female	1,5 Months	+/+	+	Conjunctivitis, Nasal Discharge
8	Shelter1-8	Female	7 Years	+/-	-	Conjunctivitis
9	Shelter1-9	Female	6 Years	+/-	-	Conjunctivitis
10	Shelter1-10	Female	3 months	+/+	-	Conjunctivitis
11	Shelter1-11	Female	2,5 Months	+/+	+	Conjunctivitis, Nasal Discharge
12	Shelter1-12	Female	8 Years	+/-	-	Conjunctivitis
13	Shelter1-13	Female	5 Years	+/-	-	Conjunctivitis
14	Shelter1-14	Female	3 Years	+/-	-	Conjunctivitis
15	Shelter1-15	Female	3 Years	+/-	-	Conjunctivitis
16	Shelter1-16	Male	4 Years	+/-	-	Conjunctivitis
17	Shelter1-17	Female	3 Years	+/-	-	Conjunctivitis
18	Shelter1-18	Female	3 Years	+/+	-	Conjunctivitis
19	Shelter2-1	Female	2,5 Months	+/+	+	Conjunctivitis, Nasal Discharge
20	Shelter2-2	Male	2,5 Months	+/+	+	Conjunctivitis, Nasal Discharge
21	Shelter2-3	Male	4 Months	+/+	+	Conjunctivitis, Nasal Discharge
22	Shelter2-4	Female	3 Months	+/+	+	Conjunctivitis, Nasal Discharge
23	Shelter3-1	Male	3 Months	+/-	+	Conjunctivitis, Nasal Discharge
24	Shelter3-2	Male	3 Months	+/-	-	Conjunctivitis
25	Shelter3-3	Female	2,5 Months	+/-	-	Conjunctivitis
26	Shelter3-4	Female	3 Months	-/+	+	Conjunctivitis, Nasal Discharge
27	Shelter4-1	Male	4 Months	+/+	+	Conjunctivitis, Nasal Discharge
28	Shelter4-2	Female	5 Months	+/-	-	Conjunctivitis
29	Shelter4-3	Male	5 Months	+/-	-	Conjunctivitis
30	Shelter4-4	Male	5 Months	+/-	+	Conjunctivitis, Nasal Discharge
31	Shelter4-5	Male	3 Years	-/+	-	Conjunctivitis
32	Shelter4-6	Female	4 Years	+/+	+	Conjunctivitis, Nasal Discharge
33	Shelter4-7	Male	6 Years	+/+	+	Conjunctivitis, Nasal Discharge
34	Shelter4-8	Male	2 Months	+/-	-	Conjunctivitis
35	Shelter4-9	Male	1,5 Months	+/+	-	Conjunctivitis
36	Shelter4-10	Male	2 Months	+/+	-	Conjunctivitis
37	Shelter4-11	Female	1,5 Months	+/-	+	Conjunctivitis, Nasal Discharge
38	Shelter4-12	Male	1,5 Months	+/-	-	Conjunctivitis
39	Shelter4-13	Female	1,5 Months	+/-	-	Conjunctivitis
40	Shelter4-14	Female	2 Months	+/+	-	Conjunctivitis
41	Shelter4-15	Male	2 Months	+/+	-	Conjunctivitis
42	Shelter4-16	Male	1,5 Months	+/-	-	Conjunctivitis
43	Shelter4-17	Male	2 Months	+/-	+	Conjunctivitis, Nasal Discharge
44	Shelter4-18	Female	3 Months	+/-	-	Conjunctivitis
				41/20 (61)	16	Totally 77 Swabs from 4 Shelters

comparative sensitivity of these primer sets is shown in Figure 1. In the PCR analyses of clinical samples, the Cfpmp1a/Cfpmp1b and Cfpmp1c/Cfpmp1d primers did not amplify any products. All the clinical samples were negative for *C. felis* in the PCR analyses.

In the PCR analyses of clinical samples, Kd1a/Kd1b (356 bp) primers and Kd2a/Kd2b (259 bp) primers amplified specific products effectively and specific bands for these primers are shown in Figure 2. Due to the proximity between the length of the amplification products, Cfpmp1a/Cfpmp1b primers (268 bp) and Kd2a/Kd2b primers (259 bp), the primers were not combined. However, in the multiplex PCR analyses with other primers, specific amplification products were obtained for *pmp* genes of *C. felis* up to 16 pg/µl and *cytb* genes of cats. The PCR products of all the simplex PCR assays and multiplex (*C. felis* and internal amplification control) assays are shown in Figure 3.

Discussion

C. felis is a bacterial agent of upper respiratory tract disease and conjunctivitis in cats. Definitive diagnosis of the agent is very important for treatment and control of the disease among cats. The agent might also have zoonotic potential. In this study, primarily the PCR assays for detection of *C. felis* were developed the *pmp1* gene of *C. felis*, then the new PCR test with host specific internal control was developed by combining these primers with cat-specific primers.

Table 2 Properties of primers used in the study

Primer Names	Target Gene GenBank Accession No	Sequences of Primers	Position of Primers in Target Gene	Length of amplicons	Reference of Primers
***Cfpmp*1a**	EF092092.1	5′-GGAGGCGATATTGTTTTCCA-3′	2764-2765	268 bp	This Study
***Cfpmp*1b**	EF092092.1	5′-AAGCAAAGCGAACTTTTCCA-3′	2994-3013		
***Cfpmp*1c**	EF092092.1	5′-GGCGATCCCTATGTTGAGAA-3′	2293-2312	155 bp	This Study
***Cfpmp*1d**	EF092092.1	5′-CCACCGAAACACCCTGTAGT-3′	2428-2447		
***Kd*1a**	AB194813.1	5′-ATGAAACTTCGGCTCCCTTC-3′	90-109	356 bp	This Study
***Kd*1b**	AB194813.1	5′-GGTTGGTGATTACGGTTGCT-3′	426-445		
***Kd*2a**	AB194813.1	5′-CGCCTTTTCATCAGTTACCC-3′	183-202	259 bp	This Study
***Kd*2b**	AB194813.1	5′-GGTGATTACGGTTGCTCCTC-3′	422-441		

The importance of PCR has increased because of the time-consuming nature of isolation of the agent in cell culture or in embryonated chicken eggs and the low sensitivity of serological assays. The detection of *C. felis* by PCR and Real-Time PCR assays is used generally with primers specific for *outher membrane protein* (*omp*) genes (Helps et al., 2003; Von Bomhard et al., 2003). Laroucau et al. (2001) showed high specifity of CpsiA/B primers designed for specific *pmp* genes of *C. abortus* PCR with positive and negative control DNA and reported that sensitivity of this primer set was equal to 5 pg/µl in the 10-fold dilutions of *C. abortus* DNA. In another study, Greco et al. (2005) reported that CpsiA/B primers set was the most sensitive primer set among four different primer sets in the PCR detection of *C. abortus.* Furthermore, the same primer set (CpsiA/B) was shown to be very sensitive and specific for PCR detection of *C. psittaci* from positive control DNA and clinical samples (Laroucau et al., 2007; Sareyyupoglu et al., 2008). Although there are reports on the use of *pmp* gene specific primers for the PCR detection of *C. abortus* and *C. psittaci*, the use of *pmp* gene specific primers for the PCR detection of *C. felis* has never been reported. In this study, the *pmp* gene specific PCR analyses were developed with two different primer sets, and *C. felis* DNA in the 10-fold dilutions of positive control were detected the Cfpmp1c/Cfpmp1d primers (1.6 pg/µl) and the Cfpmp1a/Cfpmp1b primers (16 pg/µl).

Some internal control strategies have been developed for using in PCR analysis. One of these is the addition of an external template to PCR mix (Abdulmawjood et al. 2002; Hartman et al., 2005; Lund and Madsen, 2006; Suo et al., 2010). The use of this exogenous internal control may be useful for the detection of false negative results from mixing of PCR components and thermal cycling. However, in such cases, swab samples might not contain a sufficient number of cells (Gruffydd-Jones et al., 2009). Using host DNA-specific primers is also useful for checking PCR procedure failures from sampling stage to amplification. Therefore, it was suggested that negative samples might be checked with host-specific primers to detect false negative results (Schmidt, 1997). In a study regarding the detection of upper respiratorypathogens from conjunctival swabs in cats, Helps et al. (2003) used a cat-specific 28 S ribosomal DNA gene as an internal control in the Real Time PCR

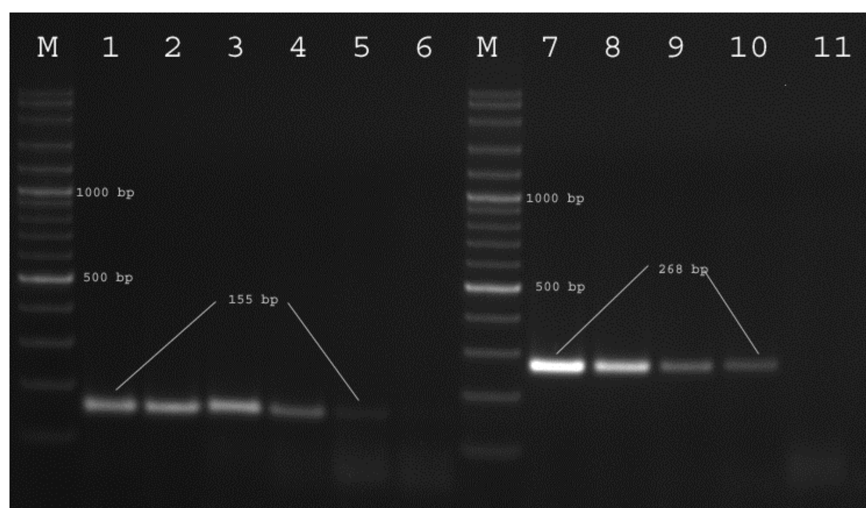

Figure 1 M; VC 100 bp Plus DNA Ladder. 1-6 Specific bands with Cfpmp1c/Cfpmp1d primers in 10-fold dilutions of *C. felis* DNA (155 bp). 7-11 Specific bands with Cfpmp1a/Cfpmp1b primers in 10-fold dilutions of *C. felis* DNA (268 bp)

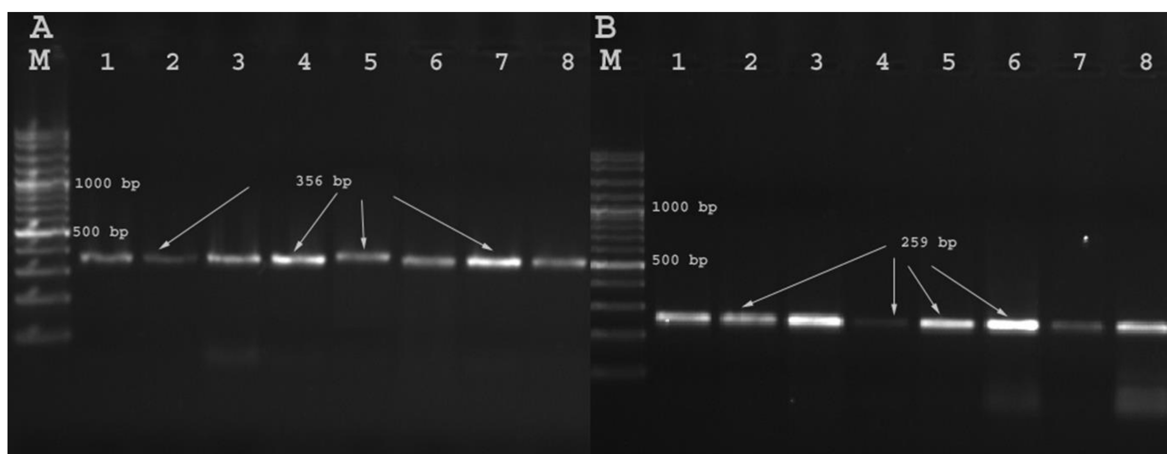

Figure 2 M; VC 100 bp Plus DNA Ladder. Section A 1-7 *F. catus cytb* gene specific bands with Kd1a/ Kd1a b primers (356 bp) in clinical samples. Section A 1-7 *F. catus cytb* gene specific bands with Kd2a/Kd2b primers (259 bp) in clinical samples

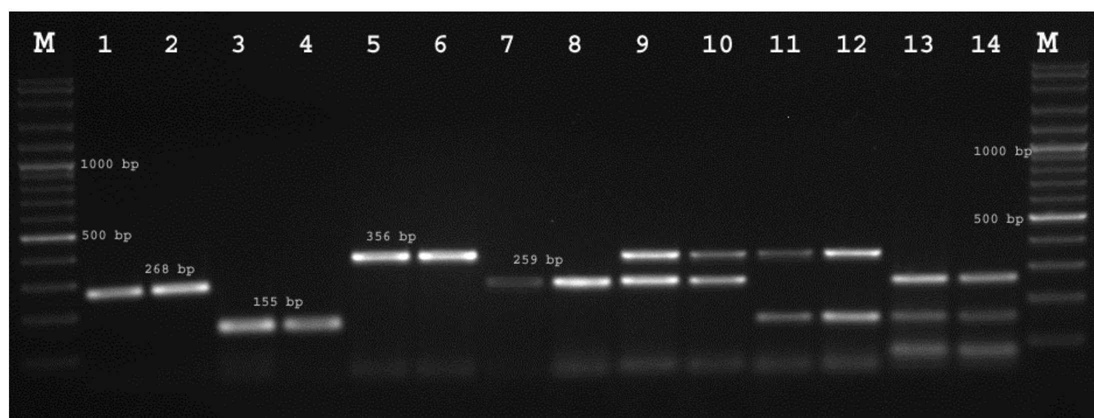

Figure 3 M; VC 100 bp Plus DNA Ladder. 1-2 *C. felis*-specific bands with Cfpmp1a/Cfpmp1b primers (268 bp). 3-4 *C. felis*-specific bands with Cfpmp1c/Cfpmp1d primers (155 bp). 5-6. *F. catus*-specific bands with Kd1a/b primers (356 bp). 7-8 *F. catus*-specific bands with Kd2a/Kd2b primers (259 bp). 9-10 Specific bands for combined Cfpmp1a/Cfpmp1b primers (268 bp) and Kd1a/Kd1b primers (356 bp). 11-12 Specific bands for combined Cfpmp1c/Cfpmp1d primers (155 bp) and Kd1a/Kd1b primers (356 bp). 13-14 Specific bands for Cfpmp1c/Cfpmp1d primers (155 bp) and Kd2a/Kd2b primers (259 bp)

detection of *C. felis*. In the current study the PCR technique was developed for the detection *C. felis* with host specific internal control that cat specific primers. The host specific internal control primers were designed *cytochrome b* genes (*cytb*) of domestic cat. These originally designed primers might have potential for multiplexing with other pathogen detection from cat samples.

In previous studies, *C. felis* was detected in 97 swab samples (18.1%) obtained from 538 cats with conjunctivitis (Helps et al., 2003), and 3.3% positivity was detected in apparently healthy cats (Di Francesco et al., 2004). In another study, Helps et al. (2005) studied 1748 cats from 218 shelters in different European countries by Real Time PCR and reported that *C. felis* was 10 per cent in the affected cats and 3 per cent in the healthy cats. Sjödahl-Essén et al. (2008) studied totally 51 (24 with ocular signs and 27 healthy control cats) cats from different origin between December 2004 and November 2005 in Sweden. The researchers reported that *C. felis* was not detected in any cats. In this study, *C. felis* was not detected in the clinical specimens, but the internal control primers amplified specific products in the samples. This situation may result from studying a small number of samples, only four shelters, and the disease in cats can be caused by other microorganisms such as viruses or other bacteria.

The designed primers allow specific and sensitive diagnosis of *C. felis*. As conventional PCR techniques have become more commonplace and cheaper than in the past, their reliability and potential uses have increased. The procedures may also be extended to the detection of other respiratory pathogens of cats, and the designed internal controls primers can be used with other bacterial agents of upper respiratory disease in cats.

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บทคัดย่อ

การพัฒนาวิธีพีซีอาร์ที่จำเพาะต่อยีน *pmp* ร่วมกับตัวควบคุมที่จำเพาะต่อโฮสต์สำหรับ *Chlamydophila felis*

Zafer Cantekin[1] Hasan Solmaz[2] Nuri Altug[3] Gamze Ozge Ozmen[1]*

Chlamydophila felis เป็นแบคทีเรียชนิดหลักในการทำให้เยื่อบุตาขาวอักเสบ และโรคของระบบทางเดินหายใจในแมว การติดต่อของโรคอาศัยการสัมผัสใกล้ชิดระหว่างแมวด้วยกัน ปฏิกิริยาลูกโซ่ของโพลีเมอเรส (PCR) มีประโยชน์ในการตรวจหาเชื้อนี้ วัตถุประสงค์ของการศึกษานี้เพื่อพัฒนาวิธี PCR ร่วมกับการเพิ่มจำนวนของตัวควบคุมภายในสำหรับการตรวจหา *C. felis* คู่ไพรเมอร์ได้ถูกออกแบบอย่างจำเพาะสำหรับยีน *polymorphic membrane protein* สำหรับ *C. felis* และยีน *cytochrome b* ของแมว ความจำเพาะ และความไวได้รับการทดสอบ ไพรเมอร์ที่จำเพาะสำหรับยีนทั้งสองจึงถูกนำไปทำมัลติเพล็กซ์ต่อ ในปฏิกิริยา PCR ที่ทำที่ค่าเจือจาง 10 เท่า DNA ของ *C. felis* ถูกตรวจพบจากไพรเมอร์ที่ได้รับการออกแบบสำหรับยีน polymorphic membrane protein ที่ค่า 1.6 pg/µl และ DNA ของแมวได้ถูกแสดงให้เห็นในทุกตัวอย่างของ PCR นอกจากนั้น ใน multiplex PCR DNA ของ *C. felis* และของแมวได้ถูกตรวจพบพร้อมกัน ไพรเมอร์ที่ออกแบบเหล่านี้และมีความจำเพาะสำหรับ *C. felis* อาจมีศักยภาพสำหรับงานวิจัยด้านการติดเชื้อ และการปล่อยเชื้อออกมาในแมว เพราะไพรเมอร์ที่จำเพาะต่อโฮสต์และเป็นการควบคุมภายในอาจมีศักยภาพสำหรับใช้ในการตรวจหาเชื้อจุลชีพอื่นได้ในแมว

คำสำคัญ: แมว *Chlamydophila felis*, multiplex polymerase chain reaction, *polymorphic membrane protein* gene

[1] *Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Microbiology, Tayfur Sokmen Campus 31000 Hatay, TURKEY*

[2] *Yüzüncü Yıl University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Campus, 65100 Van, TURKEY*

[3] *Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Internal Disease, Hatay 31000, TURKEY*

***ผู้รับผิดชอบบทความ E-mail:** *zcantekin@hotmail.com*