Ultra-fast Detection and Differentiation of *Mycoplasma haemofelis* and *Candidatus* M. Haemominutum in Korean Feral Cats by Microchip Electrophoresis with Programmed Field Strength Gradients

Kailasa S. Kumar, Hee Gu Lee,[†] Dong Jin Yoo,[‡] and Seong Ho Kang[*]

Department of Chemistry and Basic Science Research Institute, Chonbuk National University, Jeonju 561-756, Korea
[*]E-mail: shkang@chonbuk.ac.kr
[†]Cellomics Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-806, Korea
[‡]Department of Chemistry, Seonam University, Namwon 590-711, Korea
Received July 30, 2007

A microchip-based capillary gel electrophoresis (MCGE) technique was developed for the ultra-fast detection and differentiation of *Candidatus* Mycoplasma haemominutum (*Candidatus* M. haemominutum, California strain) and *Mycoplasma haemofelis* (*M. haemofelis*, Ohio strain) in Korean feral cats through the application of programmed field strength gradients (PFSG) in a conventional glass double-T microchip. The effects of the poly (ethyleneoxide) (PEO) concentration and electric field strength on the separation of DNA fragments were investigated. The PCR-amplified products of *Candidatus* M. haemominutum (202-bp) and *M. haemofelis* (273-bp) were analyzed by MCGE within 75 s under a constant applied electric field of 117.6 V/cm and a sieving matrix of 0.3% PEO (M_r 8 000 000). When the PFSG was applied, MCGE analysis generated results 6.8-times faster without any loss of resolution or reproducibility. The MCGE-PFSG technique was also applied to eleven samples selected randomly from 33 positive samples. The samples were detected and differentiated within 11 s. The analysis time of the MCGE-PFSG technique was approximately 980-times faster than that using conventional slab gel electrophoresis.

Key Words : Chip technology, Feline hemobartonellosis, Polymerase chain reaction, Programmed field strength gradients

Introduction

Haemobartonella felis (*H. felis*) is a small epicellular bacterial parasite of feline erythrocytes and the most common cause of hemolytic anemia in cats.[1] However, reports on the DNA sequences of H. *felis* suggested the existence of two morphologically and genetically distinct genotypes of H. *felis*: the larger form called the 'Ohio strain' and the smaller form called the 'California strain'.[2] These species have been reclassified and renamed as *M. haemofelis* (for the larger form) and *Candidatus* M. haemominutum (for the smaller form), and are now collectively referred to as feline hemoplasmas.[3]

Many diagnostic and epidemiological studies of H. *felis* infections in cats in various countries have been performed. Tasker and Lappin reviewed and described different types of H. *felis* in various countries, and explained the superiority of the polymerase chain reaction (PCR) in diagnosing H. *felis* in cats.[3] Moreover, the advantages of PCR for the sensitive and specific detection of *Candidatus* M. haemominutum and *M. haemofelis* from naturally infected cats by slab gel electrophoresis have been reported.[4-6] After amplication of the specific fragments, the confirmation of the PCR products is indispensable because the slab gel electrophoresis technique has notable faults, including a slow analysis rate, a high material cost and difficulty in finding heterozygous DNA.[7] In addition, this technique is labor-intensive and time-consuming. It also requires multiple sample manipulation steps. As a result, the analytical speed is very slow. Therefore, a more automated, reliable, user-friendly, ultra-fast and sensitive analytical method is needed.

Microchip electrophoresis (ME) is considered to be a revolutionary leap in analytical instrumentation. ME has many advantages for the analysis of PCR products, DNA restriction fragments, DNA sequencing and genotyping with regard to speed, reagent consumption and sensitivity.[8-10] This technique is expected to be easily integrated with other laboratories for the analysis of biological molecules, such as PCR products, immobilized enzymatic reactions and single molecule dynamics, in order to explain the sizes of the DNA molecules and solve biological problems within a short time.[11-13] Recently, our group reported the advantages of the ME with programmed field strength gradients for the ultra-fast analysis of PCR products,[14,15] this allows the rapid separation and enhanced separation efficiency of the target DNA fragments with a specific size. There has been no report on the application of ME with programmed field strength gradients to H. *felis* in Korea. To our knowledge, this is the first report of the application of the MCGE-PFSG technique to the ultra-fast detection and differentiation of *Candidatus* M. haemominutum and *M. haemofelis* in feral cats.

Experimental Section

Chemicals and reagents. A 1× TBE buffer (0.089 M Tris,

0.089 M borate and 0.002 M EDTA, pH 8.31) was prepared by dissolving a pre-mixed powder (Amerosco, Solon, OH, USA) in deionized water. A dynamic coating matrix of the microchip was made by dissolving 0.5% (w/v) polyvinylpyrrolidone (PVP, M_r 1 000 000) (Polyscience, Warrington, England) in a 1× TBE buffer containing 0.5 μg/mL ethidium bromide (EtBr) (Sigma Chemicals, St. Louis, MO, USA). The mixture was shaken for 2 min and left to stand for 2 h to remove any bubbles. The sieving matrix was made by dissolving 0.3% (w/v) of M_r 8 000 000 PEO (Sigma Chemicals, St. Louis, MO, USA) in a 1× TBE buffer containing 0.5 μg/mL EtBr, with gentle stirring overnight. A 100-bp DNA ladder (72 ng/μL) (Genepia, Korea) was used in electrophoresis.

Clinical sample collection. A total of 331 samples of feline blood, which were obtained during a Trap-Neuter-Return (TNR) program in Seong-Nam city in Korea from March to October 2006, were examined using a PCR assay. The blood samples were collected by jugular venipuncture into an ethylene diaminetetraacetic acid (EDTA) anticoagulant tube. The samples were stored at −20 °C and transported periodically to the laboratory with a cold pack for analysis.

DNA extraction and PCR amplification of the 16S rRNA. DNA extraction was carried out using a GENE ALL™ Blood Total DNA Purification kit (general bio system, Korea) according to the manufacturer's instructions. The extracted DNA was stored at −20 °C and used as a template for the PCR assay. The M. *felis* of the 16S rRNA gene was used to amplify the primers (Fig. 1). The following primers were used to amplify a 273-bp product from *M. haemofelis*: forward primer, 5'-ATG CCC CTC TGT GGG GGA TAG CCG-3'; and reverse primer, 5'-ATG GTA TTG CTC CAT CAG ACT TTC G-3'. The following primers were used to amplify a 202-bp amplicon from '*Candidatus* M. haemominutum': forward primer, 5'-CTG GGA AAC TAG AGC TTC GCG AGC-3'; and reverse primer, 5'-ATG GTA TTG CTC CAT CAG ACT TTC G-3'.[5] The PCR assay was optimized, yielding the following reaction conditions: initial denaturation for 5 min at 94 °C, followed by 35 cycles of 45 s denaturation at 94 °C, 45 s primer annealing at 58 °C, and 45 s extension at 72 °C. The template DNA (2 mL) was added to 48 mL of the reaction mixture containing 34.75 mL of sterile ultra-pure water, 5.0 mL of 10× PCR buffer, 4 mL of deoxynucleotide triphosphates (dNTPs) mixture (2.5 mM each), 1.5 mL of each primer (20 pmol/mL) and 0.75 mL of *Taq* DNA polymerase (5 U/mL, iNtRON, Korea). The *M. haemofelis* (Genebank accession no. U88563) and '*Candidatus* M. haemominutum' (Genebank accession no. U88564) DNA (kind gifts from Dr. Watanabe, University of Azabu, Japan) were included as positive controls in each PCR run to monitor the PCR reaction.

Cloning, nucleotide sequencing and phylogenetic analysis. The PCR amplicons were purified using a GFX™ PCR DNA purification kit (Amersham Biosciences, UK) according to the manufacturer's instructions for cloning and sequencing. The purified amplicons were ligated into a pGEM-T easy vector (Promega, USA) and transformed into

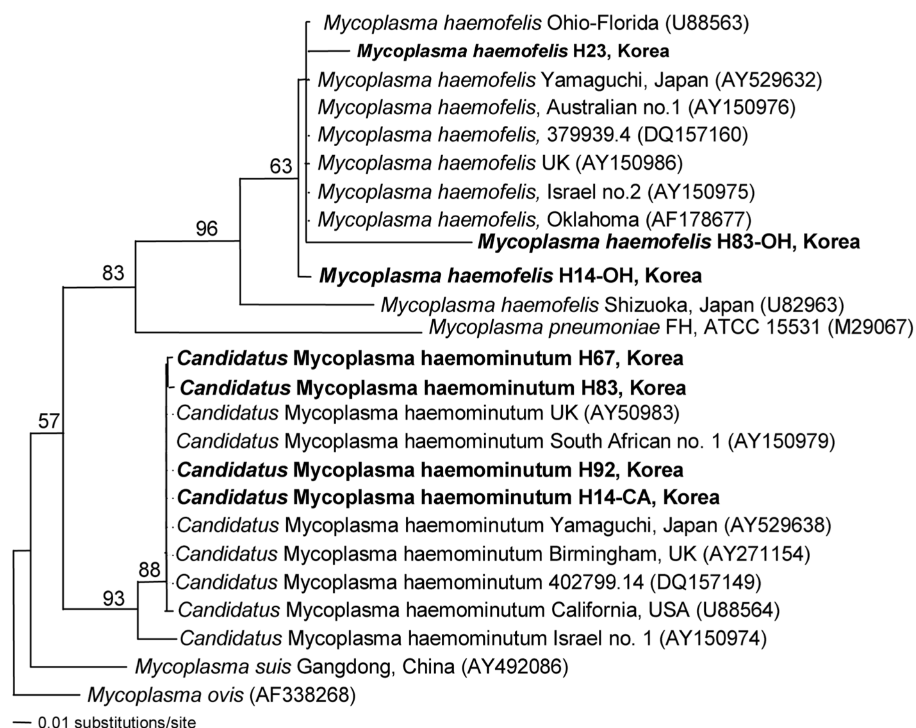

Figure 1. Phylogenetic tree based on the 16S rRNA gene sequences of hemoplasmas. The sequences of the 16S rRNA gene of *M. haemofelis* (H23, H83-OH and H14-OH) and *Candidatus* M. haemominutum (H67, H83, H92 and H14-CA) showed 98-100% and 99-100% homology with the sequences of the different isolates saved in GenBank, respectively. CA, California strain (*Candidatus* M. haemominutum, 202-bp DNA); OH, Ohio strain (*M. haemofelis*, 273-bp DNA).

TOP10F' *E. coli* competent cells. The recombinant clones were verified by colony PCR amplification, as described above and the recombinant plasmid DNA was purified using a Wizard® plus SV mini preps DNA purification system (Promega, USA). Sequencing was performed by dideoxy termination using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, USA). The sequence data was analyzed using Chromas software version. 2.31 (Technelysium, Australia). The homology searches were carried out at the National Center for Bio-technology Information (NCBI, USA) BLAST network service. The nucleotide sequences were aligned and the phylogenetic analyses were performed using the maximum-likelihood based on the obtained sequences (PAUP* 4.0b for Macintosh™).

Slab gel electrophoresis. Slab gel electrophoresis was carried out in 2% agarose gel (Sigma, St. Louis, MO, USA) with 1× TBE buffer. Two microliters of a 6× gel loading dye (bromo phenol blue:xylene cyanol FF:glycerol = 0.25:0.25: 30, %w/v) was mixed with 10 μL of each specimen. The mixture (12 μL) was loaded on 6-well gels and run at 150 V for 50 min in a SaB-Cell (Bio-RAD, USA). After electrophoresis, the gel was stained with EtBr (0.5 μg/mL) for 10 min and de-stained in nuclease free water. The samples were then photographed under UV-light using Gel Doc 2000 (Bio-RAD, USA). The presence of the 202-bp and the 273-bp DNA bands fragment of *Candidatus* M. haemominutum and *M. haemofelis*, respectively, were recorded as positive results. The size of the DNA products was determined relative to those of a size marker, *i.e.* the 100 bp DNA ladder (Bioneer, Seoul, Korea).

Microchip-based capillary gel electrophoresis. MCGE was performed on a DBCE-100 Microchip CE system (Digital Bio Technology Co., Korea) equipped with a diode-pumped solid-state laser (excitation at 532 nm and fluorescence at 605 nm; Power Technology Inc., Little Rock, AZ, USA) and a high-voltage device (DBHV-100, Digital Bio Technology Co., Korea). The microchip and schott borofloat glass were purchased from Micralyne (MCBF4-TT100, Micralyne, Canada). The microchip design, gel and sample injecting procedures have been well described in our previous papers.[14-15]

Results and Discussion

Identification of the amplified DNA by slab gel electrophoresis. Among 331 cats, thirty four (10.3%) proved positive for *Candidatus* M. haemominutum alone, 14 (4.2%) for *M. haemofelis* alone and 18 (5.4%) for both organisms. Figure 2 shows representative slab gel electropherograms of the PCR results from the 11 randomly selected samples from the samples testing positive to *H. felis*. Nucleotide sequencing of the selected amplicons confirmed the PCR products to be *Candidatus* M. haemominutum (202-bp) and *M. haemofelis* (273-bp). In all cases, the sizes of the organisms were identified based on the amplicon size after slab gel electrophoresis (Fig. 2). Within the group of cat blood with suspected haemobartonellosis, PCR was positive in 11/11; 9/11 (81.8%) were infected with *Candidatus* M. haemominutum, 5/11 (45.5%) were infected with *M. haemofelis* and 3/11 (27.3%) were infected with both.

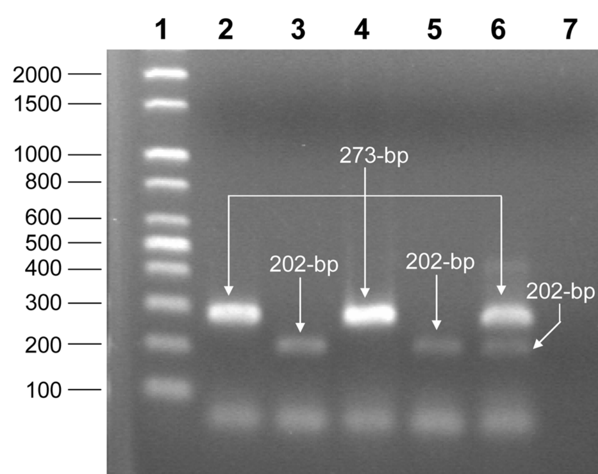

Figure 2. Representative slab gel electropherograms of the amplified PCR products for *H. felis* 16S rRNA gene from the peripheral blood of cats. The lanes are as follows: lane 1, molecular size marker (100-bp DNA ladder); lane 2, *M. haemofelis* positive control (Genbank accession no. U88563) (273-bp); lane 3, *Candidatus* M. haemominutum positive control (Genbank accession no. U88564) (202-bp); lanes 4-6, each DNA fragment of positive samples; lane 7, reagent negative control. Gel electrophoresis conditions; 2% agarose gel matrix in 1× TBE buffer; applied voltage, 150 V for 50 min; ambient temperature.

Microchip-based capillary gel electrophoresis. DNA-polymer interactions greatly influence the separation of DNA molecules. However, PEO and PVP polymers have been studied extensively in the area of DNA analysis.[16-18] Moreover, PEO and PVP have significant "self-coating" properties through adsorption to the walls.[16] Therefore, PEO and PVP were selected as the sieving and coating matrix, respectively. At first glance, the effect of the sieving matrix concentration on the separation of DNA molecules in a microchip has been studied. The effect of the sieving matrix (PEO) concentration on the separation of the DNA base pairs was examined in the range from 0.1 to 0.9%, and results were shown in Figure 3A. The above results show that all DNA fragments are separated within 120 s under an adequate resolution using the 0.3% PEO gel compared with the other PEO concentrations. Meanwhile, a long time was needed to elute the DNA fragments through the microchip channel with the higher concentration of sieving matrix. Therefore, the 0.3% PEO gel was selected as an optimum concentration of the sieving matrix.

The separation and migration time of the 200-bp and 300-bp at various electric field strengths *i.e.*, 58.8 V/cm to 294.0 V/cm were also examined (Fig. 3B). This suggests that the velocity of the DNA molecules increases with increasing electric field strength, which leads to a lower resolution and poor separation between the base pairs in the DNA molecule. On the other hand, higher voltage sources increase the temperature in the microchip, which might result in broader peaks, non-reproducible migration times and sample decom-

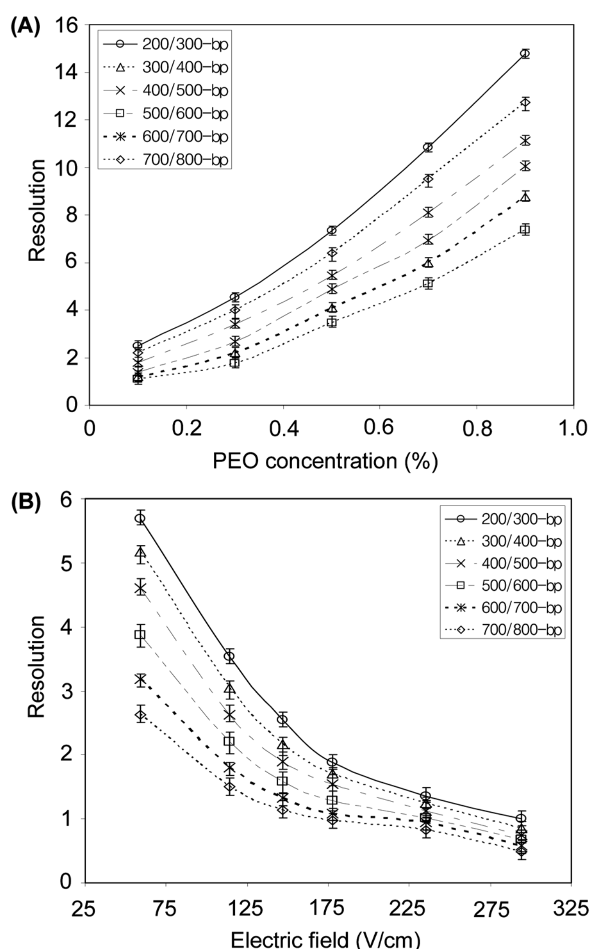

Figure 3. (A) Effect of the PEO concentration on the resolution of 200-800-bp fragments; (B) Resolution of the 200-800-bp DNA fragments as a function of the applied electric field. MCGE voltage conditions; applied separation voltage, from 29.4 to 499.8 V/cm; sample, 100-bp DNA ladder; running buffer, 1× TBE buffer (pH 8.31) with 0.5 ppm EtBr; coating matrix, 0.5% PVP (M_r 1 000 000); sieving matrix, 0.5% PEO (M_r 8 000 000).

position. This can also cause electrical discontinuity throughout the channel, which decreases the resolution and efficiency of the MCGE system. Generally, in gel electrophoresis, DNA molecules can be separated according to their size by applying an electric field. Each type of DNA molecule travels through the medium at a different rate, depending on its molecular size. However, chain entanglement in a sieving gel matrix also plays an important role in the separation of DNA molecules with different chain lengths in a gel with a given pore size.[19] This entanglement is a function of the molecular size and applied electric field, and shows irregular values in resolution.[20] The application of a high electric field strength in capillary electrophoresis leads to a shorter analysis time and a lower separation efficiency > 800-bp.[21] Interestingly, the separation efficiency of small size DNA molecules (< 300-bp) was decreased by applying a high constant field strength (Fig. 4B).

MCGE with PFSG. The main aim of this study was ultrafast detection and differentiation of feline hemobartonellosis *i.e.*, *Candidatus* M. haemominutum of 202-bp DNA and *M. haemofelis* of 273-bp DNA in cat blood by MCGE-PFSG without any loss of resolution. More study will be needed to determine the optimum electric field strength for the rapid detection and differentiation of the feline hemobartonellosis in cats. According to our previous papers,[14,15] the selected DNA fragments can be detected rapidly with adequate resolution and reduced migration time by applying a non-uniform electric field strength. In this paper, three electric field strengths were applied for the rapid separation of the 100-bp DNA ladder (DNA size marker) such as a low constant field strength (LCFS), high constant field strength (HCFS) and programmed field strength gradients (PFSG)

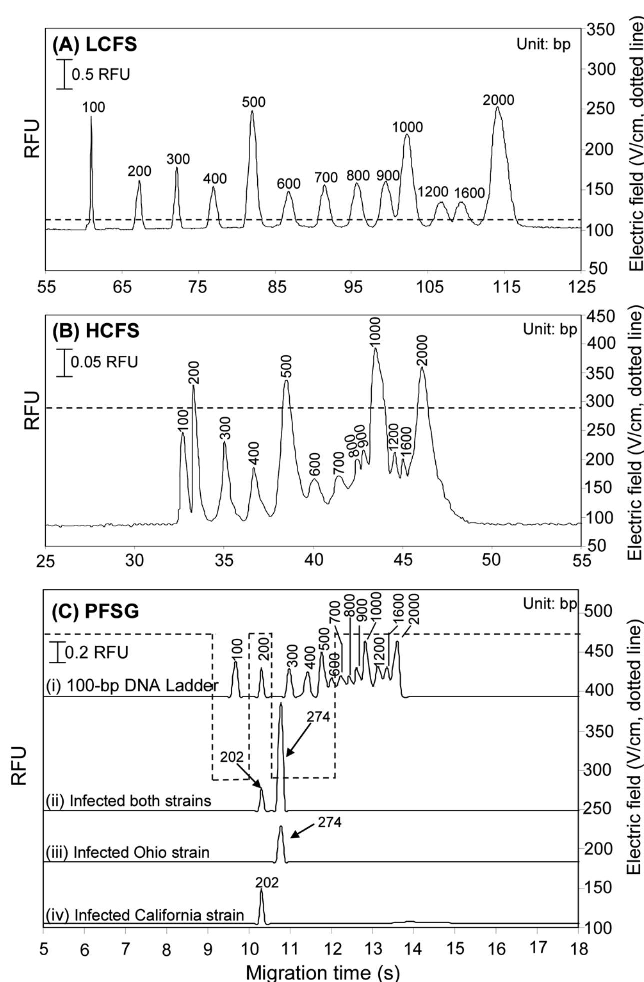

Figure 4. Representative MCGE electropherograms of the 100-bp DNA ladder fragments under: (A) LCFS, (B) HCFS and (C) MCGE-PFSG separation and detection of the PCR products in cats suspected of having feline hemobartonellosis. (i) 100-bp DNA ladder, (ii) PCR product of the amplified 202-bp and 274-bp DNA fragments from the *Candidatus* M. heamominutum and *M. haemofelis* infections, (iii) PCR product of the amplified 273-bp DNA fragment from an infection with *M. haemofelis*, (iv) PCR product of the amplified 202-bp DNA fragment from an infection with *Candidatus* M. heamominutum; MCGE condition: running buffer 1× TBE buffer (pH 8.31) with 0.5 ppm EtBr; coating matrix, 0.5% PVP (M_r 1 000 000); sieving matrix, 0.3% PEO (M_r 8 000 000); sample injection, electrokinetic injection of 56.50 V/cm for 60 s; 100-bp DNA ladder; microchip effective length, 25 mm. The dotted lines indicate the applied electric field strength.

(Fig. 4). All the DNA fragments migrated within 120 s by applying LCFS (Fig. 4A). Under the HCFS of 294.0 V/cm, all the peaks had migrated within 49 s but these had poor separation and a lower resolution (Fig. 4B). This shows that the application of HCFS in a microchip causes poor separation and a low resolution between the target base pairs in DNA molecule. In PFSG, the migration time of the 100-bp DNA fragment was reduced by applying 470.6 V/cm for 9 s and decreasing the electric field to 294.1 V/cm for 1 s (Fig. 4C). After the migration of the 100-bp DNA fragments the electric field was increased to 470.6 V/cm for 0.5 s. In this time, 200-bp migrated with adequate resolution. The electric field was then decreased to 294.1 V/cm for 1.5 s to obtain appropriate resolution between the 200-300-bp DNA fragments. After the migration of the selected base pairs, electric field was increased to 470.6 V/cm for 20 s, the resolution of the above base pairs was not considered in this study. The selected 100-300-bp DNA fragments migrated in 11 s with an adequate resolution by applying PFSG (Fig. 4C). Moreover, the resolution between the 200-bp and 300-bp DNA fragments was 2.73 ± 0.40, and the migration time was 10.30 ± 0.248 s and 10.98 ± 0.246 s at the 97% confidence interval, respectively (Table 1).

Application of the MCGE-PFSG method to the clinical samples. The applicability and utility of MCGE-PGSG for the rapid detection and differentiation of H. *felis* in cats is described. Based on the electropherograms of the different electric field strengths (Fig. 4), it was concluded that PFSG is the best method for the rapid detection and differentiation of feline hemobartonellosis in cats. In PFSG, a specific electric field was applied to the rapid separation of the specific target base pairs in the DNA ladder. This is the main advantage of PFSG over constant field strength gradients. In the PFSG electropherogram (Fig. 4C), all the target base pairs migrated within 11 s and the resolution of the target base pairs was 2.73 ± 0.40. Fig. 4C and Table 1 highlight the advantage of the PFSG method with regard to the migration time and resolution of the selected base pairs. Therefore, PFSG was selected in this study because the aim of this study is the ultra-fast detection and differentiation of haemobartonellasis (202-bp DNA for *Candidatus* M. haemominutum) and (273-bp DNA for *M. haemofelis*) in feral cats within 11 s (Fig. 4C).

Table 1. Comparison of the PFSG method with LCFS and HCFS for the ultra-fast separation of the 200-bp and 300-bp DNA fragments in a 100-bp DNA ladder

Applied electric field (V/cm)	Migration time (s)		R_s[a]
	200-bp	300-bp	
LCFS	67.40	72.80	4.52 ± 0.139
HCFS	33.20	35.04	1.81 ± 0.321
PFSG	10.30	10.98	2.73 ± 0.40

Applied separation voltage, 117.6 V/cm for LCFS; 294.0 V/cm for HCFS; applied separation voltage, 470.6 V/cm for 9 s, 294.1 V/cm for 1 s, 470.6 V/cm for 0.5 s, 294.1 V/cm for 1.5 s, 470.6 V/cm for 20 s, run buffer 1× TBE buffer (pH 8.31) with 0.5 ppm of EtBr; coating matrix, 0.5% PVP (M_r 1 000 000); sieving matrix, 0.3% PEO (M_r 8 000 000) at an effective length of 25 mm. [a]R_s (resolution between 200-bp and 300-bp DNA fragments) = $\Delta t/W_{ave}$ (Δt is the difference in migration time between two adjacent peaks; W_{ave} is the average peak width of baseline).

In this study, the PCR results of traditional slab gel electrophoresis and MCGE were compared to determine the potential of microchip technology for the rapid differentiation of *Candidatus* M. haemominutum (202-bp) and *M. haemofelis* (273-bp) in feral cats. Although traditional slab gel electrophoresis is a clearly established method for examining PCR-amplified DNA fragments, there is evidence that capillary electrophoresis provides faster separation with a higher resolution.[22] MCGE has attracted increasing interest as an alternative system for the analysis of DNA molecules.[23] Electrophoretic analysis of the PCR products for the detection and differentiation of feline hemobartonellosis was carried out in a 2% agarose slab gel and in 0.3% PEO in the microchip (Figs. 2 and 4C). It should be noted that the microchip readout shows the increasing size of the PCR product from left to right, and the results obtained by the microchip is essentially the same as those obtained in the agarose gel (Fig. 4C). The detection and differentiation of the PCR products in MCGE-PFSG was much easier, and the procedure was much faster than conventional slab gel electrophoresis. The reproducibility of the method was confirmed by an analysis of the 100-bp DNA ladder, which clearly demonstrated the superiority of MCGE over the slab gel method with regard to time and sensitivity. The half-peak widths and number of theoretical plate values were 0.85 s and 8900 for 200-bp DNA and 0.61 s and 6700 for 300-bp DNA, respectively.

When the MCGE-PFSG technique is applied to the analysis of the PCR products from the blood of feral cats infected with Haemobartonella felis, there was a 100% correlation between these results and those obtained using conventional slab gel electrophoresis (Table 2). However, there is considerable difficulty in identifying heterozygous

Table 2. Comparison of the MCGE-PFSG method with conventional slab gel electrophoresis for the detection of PCR products from the blood of feral cats infected with Haemobartonella felis

Case number	Kinds of strain in cat blood	MCGE-PFSG		Slab gel electrophoresis	
		CA[a]	OH[b]	CA[a]	OH[b]
H14	CA/OH	+	+	+	+
H23	CA/OH	ND[c]	+	ND[c]	+
H58	CA/OH	+	+	+	+
H61	CA/OH	+	ND[c]	+	ND[c]
H64	CA/OH	+	ND[c]	+	ND[c]
H65	CA	+	ND[c]	+	ND[c]
H66	CA	+	ND[c]	+	ND[c]
H67	CA	+	ND[c]	+	ND[c]
H70	OH	ND[c]	+	ND[c]	+
H83	CA/OH	+	+	+	+
H92	CA	+	ND[c]	+	ND[c]

[a]CA: California strain (*Cadidatus* M. haemominutum, 202-bp DNA). [b]OH: Ohio strain (*M. haemofelis*, 273-bp DNA). [c]ND: Not detected.

DNA molecules with slab gel electrophoresis.[7] Finally, MCGE-PFSG technology is a good alternative to conventional methods for the ultra-fast detection and differentiation of feline hemobartonellosis in cats as well as other clinical diagnostics.

Conclusions

MCGE-PFSG was introduced in order to increase the separation efficiency between the target DNA fragments with a specific size and reduce the migration time for the rapid detection and differentiation of feline hemobartonellosis through the application of a non-uniform electric field in MCGE system. PFSG vastly improves the resolution ($R_s > 2.7$) and migration time (< 11 s) for the separation of specific size DNA fragments (*i.e*., 200-bp and 300-bp) compared with the HCFS. The differentiation of feline hemobartonellosis *i.e*., *Candidatus* M. haemominutum (202-bp) and *M. haemofelis* (273-bp) can be accomplished with greater speed and simplicity using MCGE by applying PFSG than the currently available technologies. The sensitivity, speed, simplicity and reagent consumption of the MCGE with PFSG technology holds great potential for extrapolation to the detection and differentiation of mutations in DNA with a similar molecular biology, which makes it attractive for use in a clinical diagnostic laboratory.

Acknowledgements. The authors wish to thank Dr. Jinho Park for supplying the real samples. This work was supported by a grant from the Post-Doc. Program, Chonbuk National University (2006), Jeonju, South Korea and by a grant from Korea Ministry of Science and Technology (M1053608003-05N3608-00310).

References

1. Flint, J. C.; Roepke, M. H.; Jensen, R. *Am. J. Vet. Res.* **1958**, *19*, 164.
2. Berent, L. M.; Messick, J. B.; Cooper, S. K. *Am. J. Vet. Res.* **1998**, *59*, 1215.
3. Tasker, S.; Lappin, M. R. *J. Feline Med. Surg.* **2002**, *4*, 3.
4. Inokuma, H.; Taroura, S.; Okuda, M.; Hisasue, M.; Itamoto, K.; Une, S.; Nakaichi, M.; Taura, Y. *J. Vet. Med. Sci.* **2004**, *66*, 1017.
5. Watanabe, M.; Hisasue, M.; Hashizaki, K.; Furuichi, M.; Ogata, M.; Hisamatsu, S.; Ogi, E.; Hasegawa, M.; Tsuchiya, R.; Yamada, T. *J. Vet. Med. Sci.* **2003**, *65*, 1111.
6. Kewish, E. K.; Appleyard, D. G.; Myers, L. S.; Kidney, A. B.; Jackson, L. M. *Can. Vet. J.* **2004**, *45*, 749.
7. Tian, H.; Brody, L. C.; Landers, J. P. *Genome Research* **2000**, *10*, 1403.
8. Dolnik, V.; Liu, S. *J. Sep. Sci.* **2005**, *28*, 1994.
9. Kang, S. H.; Jang, S.; Park, S.-G.; *Bull. Korean Chem. Soc.* **2006**, *27*, 1346.
10. Jang, S.; Cho, K.; Chae, J.-S.; Kang, S. H. *Bull. Korean Chem. Soc.* **2004**, *25*, 757.
11. Sung, W. C.; Lee, G. B.; Tzeng, C. C.; Chen, S. H. *Electrophoresis* **2001**, *22*, 1188.
12. Baba, Y. *J. Chromatogr. B* **1996**, *687*, 271.
13. Ronai, Z.; Barta, C.; Sasvari-Szekely, M.; Guttman, A. *Electrophoresis* **2001**, *22*, 294.
14. Kang, S. H.; Park, M.; Cho, K. *Electrophoresis* **2005**, *26*, 3179.
15. Kim, Y. J.; Chae, J. S.; Chang, J. K.; Kang, S. H. *J. Chromatogr. A* **2005**, *1083*, 179.
16. Fung, E. N.; Yeung, E. S. *Anal. Chem.* **1995**, *67*, 1913.
17. Chang, H. T.; Yeung, E. S. *J. Chromatogr. B* **1995**, *669*, 113.
18. Gao, Q.; Yeung, E. S. *Anal. Chem.* **1998**, *70*, 1382.
19. Smisek, D. L.; Hoagland, D. A. *Science* **1990**, *248*, 1221.
20. Demana, T.; Lanan, M.; Morris, M. D. *Anal. Chem.* **1991**, *63*, 2795.
21. Guttman, A.; Wanders, B.; Cooke, N. *Anal. Chem.* **1992**, *64*, 2348.
22. Dolnik, V.; Liu, S. *J. Sep. Sci.* **2005**, *28*, 1994.
23. Oda, R. P.; Bush, V. J.; Landers, J. P. *Hand Book of Capillary Electrophoresis*, 2nd ed.; CRC Press: Boca Raton, 1997; p 673.