Contract No. MRG5680033

Final Report

Project Title Investigation of culture-negative bacterial infections; stealth pathogens in pet animals using broad range PCR detection

Researcher	Institute
1. Gunn Kaewmongkol	Kasetsart University, Faculty of Veterinary Medicine
2. Assoc Prof Sathaporn Jittapalapong	Kasetsart University, Faculty of Veterinary Medicine
3. Prof Stan Fenwick	Department of Infectious Disease and Global Health, Cummings School of Veterinary Medicine, Tufts University, Boston, USA

This project granted by the Thailand Research Fund

Abstract

Project Code: MRG5680033

Project Title: Investigation of culture-negative bacterial infections; stealth pathogens in pet animals using broad range PCR detection

Investigator: Gunn Kaewmongkol, DVM, MS, PhD

E-mail Address: gunn_kaew@yahoo.com, fvetgunn@ku.ac.th

Project Period: 3 June 2013 – 2 June 2015

Abstract:

This is a two-year study; the aim of the first year is to develop improved broad-range nested PCR methods to detect bacterial DNA in culture-negative specimens in pet animals presented at Veterinary teaching hospital, Kasetsart University, Bangkok, Thailand. The analytical sensitivity of the nested broad-range PCR targeting the 16S rRNA gene was determined by testing of ten serial 10-fold dilutions which ranged from 10^{6}-10^{-3} bacterial colony forming unit (CFU/ml) The standard serial dilutions were prepared from 6 known bacterial genus and species including *Escherichia coli, Staphylococcus* species, *Streptococcus* species, *Pseudomonas* species, *Klebsiella* species and *Proteus* species. According to the analytical sensitivity, the lowest dilution of bacterial culture detected by this tool was 10^{-3} CFU/ml in all bacteria prepared for this study. The aim of the second year is to apply this new technique for diagnosis of bacterial infections in a variety of clinical specimens that all samples were negative results from conventional bacterial culture methods.

Urinary tract infections and bacterial prostatitis are commonly diagnosed and treated by broad-range antibiotics prior to performing of standard bacterial culture and antibiotic sensitivity test. Consequently, conventional bacterial culture techniques frequently fail to isolate pathogenic bacteria from most specimens of these cases. A broad-range nested PCR detection developed in the recent study was applied for the diagnosis of urogenital tract infections in dogs and cats which were pretreated with antibiotics and pathogenic organisms were not isolated from their specimens using standard culture techniques. Total of 118 urine and 46 semen specimens were included in this study that these specimens were negative results from conventional bacterial culture methods. The new

nested PCR detected bacterial 16S rDNA in 25/46 semen specimens (54%) and 51/118 urine specimens (43%).

The uncommon organisms were detected in cerebrospinal fluid (CSF) samples from the CNS patients that those CSF specimens were negative for the conventional bacterial culture techniques. This part reported and discussed rare cases of *Ehrlichia canis* meningoencephalitis in dogs without thrombocytopenia. The organism has been a cause of high morbidity and mortality in dogs in Southeast Asia since being identified in military dogs during the Vietnam War in the late 1980s. However, published reports on this organism in this region, both clinical manifestations and genetic studies, are very limited. According to the uncommon clinical signs and hematological findings of these cases, the veterinary practitioners who treated such the cases excluded *E. canis* infection from the differential diagnosis and consequently, there was no specific ELISA test and specific drug for *E. canis*. Without our report, there could be the same cases occurring again and most veterinary practitioners will not include *E. canis* infection in the cases of meningoencephalitis in dogs without thrombocytopenia. This report demonstrated the advantage of this technique that should be performed routinely in veterinary laboratories, particularly in samples from CNS patients with CSF culture-negative results.

This study also investigated bacterial infections in clinical abscesses from 20 cases using the new broad range PCR technique. Conventional bacterial culture isolated bacteria in 9 abscesses from a total of 20 samples. Interestingly, the PCR detected bacterial DNA in 17 abscesses and fastidious obligate anaerobic bacteria were detected in 10 samples. In contrast to the PCR results, anaerobic bacterial culture could not isolate the anaerobic bacteria in all samples. This broad range PCR was able to detect dominant anaerobic bacteria in abscesses that these dominant bacteria were overlooked by the conventional bacterial culture.

Eighty one anemic dogs with unknown causes were screened for hemotropic bacterial infections using this broad-range PCR technique. The new nested PCR detected bacterial 16S rDNA in 10/81 blood specimens (12.34%). Three types of organisms were detected in these blood samples including *Ehrlichia canis* (5), *Anapalsma platys* (3) and hemotropic *Mycoplasma* spp. (2).

The detection of bacterial 16S rDNA in various clinical specimens suggests the advantage of the broad range PCR technique, developed in this study, for the diagnosis of culture-negative bacterial infections in pet animals. In conclusion, this broad range PCR technique should be recommended in veterinary laboratories, particularly in inflammatory samples with culture-negative results. However, diversity of bacterial species could be found in one clinical sample instead of single species reported in our study. The PCR products amplified in this study could be applied for other next generation sequencing techniques in order to confirm the theory of imbalance bacteria population in clinical specimens.

บทคัดย่อ:

จากโครงการวิจัย 2 ปีครั้งนี้ วัตถุประสงค์ในปีแรกคือการพัฒนาเทคนิคพีซีอาร์ชนิดวงกว้างเพื่อการตรวจหาสารพันธุกรรมของแบคทีเรียหลากหลายชนิดจากตัวอย่างที่ให้ผลลบต่อการเพาะเชื้อแบบเดิม จากตัวอย่างที่เก็บจากสัตว์ที่เข้ารับการตรวจรักษา ณ โรงพยาบาลสัตว์ คณะสัตวแพทยศาสตร์ มหาวิยาลัยเกษตรศาสตร์ วิทยาเขตบางเขน ทำการวิเคราะห์ความไวของเทคนิคใหม่นี้ โดยทดสอบกับ สารละลายเพาะเลี้ยงเชื้อแบคทีเรีย โดยเจือจาง 10 เท่า ตั้งแต่ความเข้มข้น 10^{6} -10^{-3} CFU/ml สารละลายเจือจางเหล่านี้เตรียมจากแบคทีเรีย 6 ชนิดคือ *Escherichia coli Staphylococcus* spp *Streptococcus* spp *Pseudomonas* spp *Klebsiella* spp และ *Proteus* spp จากการทดสอบความไวพบว่าระดับสารละลายเจือจางต่ำที่สุดที่สามารถตรวจได้คือ 10^{-3} CFU/ml ในแบคทีเรียทุกชนิดที่ทดสอบในการศึกษาครั้งนี้ จุดประสงค์ของโครงการในปีที่สองคือการนำเทคนิคที่พัฒนาสำเร็จแล้ว ไปประยุกต์ใช้ในการตรวจการติดเชื้อแบคทีเรียในตัวอย่างทางคลินิก โดยตัวอย่างเหล่านั้นให้ผลลบต่อการเพาะเชื้อแบบปกติ

การติดเชื้อแบคทีเรียในทางเดินปัสสาวะและต่อมลูกหมากในสุนัข มักจะถูกวินิจฉัยและได้รับการรักษาเบื้องต้นโดยการให้ยาปฏิชีวนะแบบวงกว้าง ก่อนการเพาะเชื้อแบคทีเรียและทดสอบความไวของยา ผลที่ตามมาคือความล้มเหลวที่จะวินิจฉัยหาเชื้อสาเหตุโดยการเพาะเชื้อแบบปกติจากตัวอย่างในสัตว์ป่วยเหล่านี้ การศึกษาในครั้งนี้ได้นำวิธีพีซีอาร์แบบวงกว้างที่พัฒนาขึ้นใหม่มาใช้ตรวจหาเชื้อแบคทีเรียในตัวอย่างปัสสาวะและน้ำเชื้อที่ให้ผลลบต่อการเพาะเชื้อแบบปกติ และถูกสงสัยว่าเป็นการอักเสบจากการติดเชื้อแบคทีเรีย ทำการทดสอบในตัวอย่างปัสสาวะทั้งหมด 118 ตัวอย่าง และตัวอย่างน้ำเชื้อทั้งหมด 46 ตัวอย่าง ให้ผลบวกต่อวิธีพีซีอาร์ทั้งหมด 51 ตัวอย่างจาก 118 ตัวอย่างของปัสสาวะ (54%) และ พบผลบวก 25 ตัวอย่างจาก 46 ตัวอย่างของน้ำเชื้อ (43%)

ทำการตรวจหาเชื้อแบคทีเรียในน้ำไขสันหลังของสุนัขที่ป่วยด้วยอาการทางระบบประสาท โดยตัวอย่างน้ำไขสันหลังให้ผลลบต่อการเพาะเชื้อแบบปกติ ผลตรวจพบเชื้อที่ไม่พบบ่อยและมีรายงานน้อยคือเชื้อแบคทีเรีย *Ehrlichia canis* ที่ทำให้เกิดอาการสมองและเยื่อหุ้มสมองอักเสบ โดยไม่พบภาวะเกล็ดเลือดต่ำ ซึ่งถือเป็นค่าความเปลี่ยนแปลงของเลือดที่ใช่เป็นข้อบ่งชี้สำคัญในการวินิจฉัยการติดเชื้อชนิดนี้ เชื้อ *Ehrlichia canis* เป็นเชื้อที่ก่อโรคและสามารถทำให้สุนัขเสียชีวิตได้สูง ซึ่งถูกรายงานไว้ตั้งแต่ในช่วงทศวรรษที่ 80 ในช่วงสงครามเวียดนามที่มีการนำสุนัขสงครามเข้ามาปฏิบัติภารกิจสงครามในเขตอุษาคเนย์ แต่จนถึงปัจจุบันกลับมีรายงานทางคลินิกและพันธุกรรมของเชื้อชนิดนี้ในประเทศไทยและภูมิภาคนี้ค่อนข้างน้อย จากสัตว์ป่วยที่รายงานในครั้งนี้ไม่พบความผิดปกติของเกล็ดเลือดซึ่งเป็นตัวบ่งชี้สำคัญในการวินิจฉัย สัตวแพทย์ผู้ทำการรักษาจึงไม่ได้รวมสาเหตุการติดเชื้อ *E. canis* เข้ามาในแผนการวินิจฉัยแยกโรค จึงไม่ได้ทำการตรวจยืนยันการติดเชื้อโดยเทคนิกทางภูมิคุ้มกัน ELISA ซึ่งเป็นการตรวจที่ทำเป็นปกติในการยืนยันการติดเชื้อชนิดนี้ เมื่อไม่ได้รวมการติดเชื้อ *E. canis* อยู่ในแผนการวินิจฉัย ดังนั้นจึงไม่มีการให้ยารักษาที่จำเพาะต่อเชื้อชนิดนี้ หากไม่มีการนำเทคนิกที่พัฒนาขึ้นในการศึกษาครั้งนี้มาใช้ และไม่มีการเผยแพร่ผลงานวิจัยนี้ สุนัขที่มีอาการและการติดเชื้อแบบเดียวกันนี้โดยไม่พบภาวะเกล็ดเลือดต่ำ สัตวแพทย์ผู้รักษาก็จะไม่รวมการติดเชื้อ *E. canis* เข้ามาในแผนการวินิจฉัยแยกโรคดังที่ทำมา

แต่เดิม ดังนั้นการศึกษานี้แสดงให้เห็นข้อได้เปรียบของเทคนิคใหม่ที่พัฒนาขึ้น ซึ่งควรนำมาใช้ในวิธีการตรวจประจำ โดยเฉพาะกรณีสุนัขที่มีอาการทางระบบประสาทที่สงสัยสาเหตุจากติดเชื้อ และน้ำไขสันหลังให้ผลลบต่อการเพาะเชื้อแบบปกติ

การศึกษานี้ได้ทำการตรวจการติดเชื้อแบคทีเรียในตัวอย่างฝีหนองทั้งหมด 20 ราย จากผลการเพาะเชื้อปกติพบการติดเชื้อแบคทีเรียใน 9 รายจากทั้งหมด 20 ราย (45%) แต่จากการใช้เทคนิคใหม่ที่พัฒนาขึ้น พบเชื้อแบคทีเรียทั้งหมด 17 รายจาก 20 ราย (85%) จากผลบวก 17 ราย พบการติดเชื้อแบคทีเรียชนิดไม่พึ่งพาอากาศ (anaerobic bacteria) ทั้งหมด 10 ตัวอย่าง ในทางกลับกัน การเพาะเชื้อปกติไม่สามารถตรวจพบเชื้อ anaerobes จากตัวอย่างเดียวกันนี้ เทคนิคใหม่ที่พัฒนาขึ้นสามารถตรวจพบแบคทีเรียชนิดหลักที่เป็น anaerobes ในตัวอย่างฝีหนอง โดยเชื้อ anaerobes เหล่านี้ไม่สามารถตรวจพบได้โดยวิธีเพาะเชื้อแบบปกติ

เทคนิคใหม่ที่พัฒนาขึ้นในครั้งยังได้นำไปตรวจหาเชื้อแบคทีเรียในตัวอย่างเลือดสุนัขทั้งหมด 81 ตัวอย่าง ที่มีภาวะโลหิตจางและสงสัยสาเหตุโลหิตจางจากการติดเชื้อ แต่ยังไม่สามารถระบุสาเหตุของโลหิตจางได้ ตรวจพบผลบวก 10 ตัวอย่างจากทั้งหมด 81 ตัวอย่าง (12.34%) พบเชื้อ 3 ชนิดคือ *Ehrlichia canis* 5 ตัวอย่าง *Anaplasma platys* 3 ตัวอย่าง และ hemotropic *Mycoplasma* 2 ตัวอย่าง

การตรวจพบการติดเชื้อแบคทีเรียจากหลากหลายตัวอย่างในการศึกษาครั้งนี้แสดงให้เห็นข้อได้เปรียบของเทคนิคพีซีอาร์แบบวงกว้างที่พัฒนาขึ้น สำหรับการวินิจฉัยการติดเชื้อที่ให้ผลลบต่อการเพาะเชื้อแบบปกติในสัตว์เลี้ยง จากผลการศึกษาโดยสรุปคือ ควรมีการนำเทคนิคนี้หรือเทคนิคในแนวคิดแบบเดียวกันนี้ มาปรับใช้ในห้องปฏิบัติการทางสัตวแพทย์ โดยเฉพาะตัวอย่างจากการอักเสบที่ให้ผลลบต่อการเพาะเชื้อแบบปกติ อย่างไรก็ตามมีการรายงานการติดเชื้อแบคทีเรียหลากหลายชนิดใน 1 ตัวอย่างทางคลินิค โดยไม่ได้เป็นการติดเชื้อเดี่ยวในแบบที่รายงานในการศึกษานี้ ผลผลิตจากพีซีอาร์ในการศึกษาครั้งนี้สามารถนำไปตรวจเพิ่มเติมด้วยเทคนิคอื่นๆได้ทันที (Next generation DNA sequencing techniques; Pyrosequencing) เพื่อยืนยันทฤษฎีการก่อโรคจากการติดเชื้อแบคทีเรีย ที่เกิดจากการเสียสมดุลย์ของประชากรเชื้อแบคทีเรียเจ้าบ้าน ที่แต่เดิมไม่ใช่เชื้อก่อโรค

Keywords: culture-negative bacteria, broad-range PCR, clinical specimen, pet animals

Final report content:

1. Abstract

This is a two-year study; the aim of the first year is to develop improved broad-range nested PCR methods to detect bacterial DNA in culture-negative specimens in pet animals presented at Veterinary teaching hospital, Kasetsart University, Bangkok, Thailand. The analytical sensitivity of the nested broad-range PCR targeting the 16S rRNA gene was determined by testing of ten serial 10-fold dilutions which ranged from 10^{6}-10^{-3} bacterial colony forming unit (CFU/ml) The standard serial dilutions were prepared from 6 known bacterial genus and species including *Escherichia coli, Staphylococcus* species, *Streptococcus* species, *Pseudomonas* species, *Klebsiella* species and *Proteus* species. According to the analytical sensitivity, the lowest dilution of bacterial culture detected by this tool was 10^{-3} CFU/ml in all bacteria prepared for this study. The aim of the second year is to apply this new technique for diagnosis of bacterial infections in a variety of clinical specimens that all samples were negative results from conventional bacterial culture methods.

Urinary tract infections and bacterial prostatitis are commonly diagnosed and treated by broad-range antibiotics prior to performing of standard bacterial culture and antibiotic sensitivity test. Consequently, conventional bacterial culture techniques frequently fail to isolate pathogenic bacteria from most specimens of these cases. A broad-range nested PCR detection developed in the recent study was applied for the diagnosis of urogenital tract infections in dogs and cats which were pretreated with antibiotics and pathogenic organisms were not isolated from their specimens using standard culture techniques. Total of 118 urine and 46 semen specimens were included in this study that these specimens were negative results from conventional bacterial

culture methods. The new nested PCR detected bacterial 16S rDNA in 25/46 semen specimens (54%) and 51/118 urine specimens (43%).

The uncommon organisms were detected in cerebrospinal fluid (CSF) samples from the CNS patients that those CSF specimens were negative for the conventional bacterial culture techniques. This part reported and discussed rare cases of *Ehrlichia canis* meningoencephalitis in dogs without thrombocytopenia. The organism has been a cause of high morbidity and mortality in dogs in Southeast Asia since being identified in military dogs during the Vietnam War in the late 1980s. However, published reports on this organism in this region, both clinical manifestations and genetic studies, are very limited. According to the uncommon clinical signs and hematological findings of these cases, the veterinary practitioners who treated such the cases excluded *E. canis* infection from the differential diagnosis and consequently, there was no specific ELISA test and specific drug for *E. canis.* Without our report, there could be the same cases occurring again and most veterinary practitioners will not include *E. canis* infection in the cases of meningoencephalitis in dogs without thrombocytopenia. This report demonstrated the advantage of this technique that should be performed routinely in veterinary laboratories, particularly in samples from CNS patients with CSF culture-negative results.

This study also investigated bacterial infections in clinical abscesses from 20 cases using the new broad range PCR technique. Conventional bacterial culture isolated bacteria in 9 abscesses from a total of 20 samples. Interestingly, the PCR detected bacterial DNA in 17 abscesses and fastidious obligate anaerobic bacteria were detected in 10 samples. In contrast to the PCR results, anaerobic bacterial culture could not isolate the anaerobic bacteria in all samples. This broad range PCR was able to detect dominant anaerobic bacteria in abscesses that these dominant bacteria were overlooked by the conventional bacterial culture.

Eighty one anemic dogs with unknown causes were screened for hemotropic bacterial infections using this broad-range PCR technique. The new nested PCR detected bacterial 16S rDNA in 10/81 blood specimens (12.34%). Three types of organisms were detected in these blood samples including *Ehrlichia canis* (5), *Anapalsma platys* (3) and hemotropic *Mycoplasma* spp. (2).

The detection of bacterial 16S rDNA in various clinical specimens suggests the advantage of the broad range PCR technique, developed in this study, for the diagnosis of culture-negative bacterial infections in pet animals. In conclusion, this broad range PCR technique should be recommended in veterinary laboratories, particularly in inflammatory samples with culture-negative results. However, diversity of bacterial species could be found in one clinical sample instead of single species reported in our study. The PCR products amplified in this study could be applied for other next generation sequencing techniques in order to confirm the theory of imbalance bacteria population in clinical specimens.

2. Executive summary

The aim of this study was to develop improved broad-range nested PCR methods to detect bacterial DNA in culture-negative specimens in pet animals presented at Veterinary teaching hospital, Kasetsart University, Bangkok, Thailand. The impetus of this work was initiated because the regularly failures of conventional bacterial isolations have occurred in veterinary practices. In addition, these situations are further complicated by the lack of suitable tools for the detection of culture-negative-bacteria in various clinical specimens. Furthermore, the optimizations of this broad range nested-PCR were performed and the sensitivity of this tool was analyzed in order to clarify the minimal limit for the detection of bacterial DNA. The analytical sensitivity of the nested broad-range PCR targeting the 16S rRNA gene was

determined by testing of ten serial 10-fold dilutions which ranged from 10^{6}-10^{-3} bacterial colony forming unit (CFU/ml) and concentration of DNA extracted from each dilution was measured by the Nanodrop^{R} spectrophotometer. The standard serial dilutions were prepared from 6 known bacterial genus and species including *Escherichia coli, Staphylococcus* species, *Streptococcus* species, *Pseudomonas* species, *Klebsiella* species and *Proteus* species. According to the analytical sensitivity, the lowest dilution of bacterial culture detected by this tool was 10^{-3} CFU/ml in all bacteria prepared for this study. All PCR products produced by this tool were confirmed by DNA sequencing and these sequencing results demonstrated the correlations between genotypic status of all known bacteria and phenotypic status which included bacterial morphology and biochemical test. DNA extracted from each dilution was measured and DNA concentrations of the lowest dilution (10^{-3} CFU/ml) ranged from 0.5-1.9 ng/µl.

We applied the broad-range nested PCR detection developed in the recent study for the diagnosis of urogenital tract infections in dogs and cats which were pretreated with antibiotics and pathogenic organisms were not isolated from their specimens using standard culture techniques. Total of 118 urine and 46 semen specimens were included in this study that these specimens were negative results from conventional bacterial culture methods. DNA sequence similarity was analyzed using the Basic Local Alignment Search Tool (BLAST) to find related sequences in GenBank database. The new nested PCR detected bacterial 16S rDNA in 25/46 semen specimens (54%) and 51/118 urine specimens (43%). The BLAST algorithm revealed close related organisms to these 16S rDNA sequences including *Berkholderia* spp., *Sphingomonas* spp., *Stenotrophomonas* spp., *Pseudomonas* spp., *Enterobacter* spp., *Enterococcus* spp., *Mycoplasma* spp., *Ureaplasma* spp., *Corynebacterium* spp., *Citrobacter* spp.,

Cloacibacterium spp., *Staphylococcus* spp., *Leptotrichia* spp., *Moraxella* spp., *Comamonas* spp., *Actinobacillus* spp., *Klebsiella* spp. and *Acinetobacter* spp.

We also report rare cases of *Ehrlichia canis* meningoencephalitis in dogs without thrombocytopenia. Bacterial culture failed to isolate microorganisms in the cerebrospinal fluid which were subsequently screened for bacterial infection using the broad range 16S rRNA PCR developed as part of our study. Sequencing of 16S rRNA gene products revealed the organism closely related to *Ehrlichia canis*. Phylogenetic analysis of the *gp36* gene demonstrated an association between the *E. canis* genotype detected in the CSF and *E. canis* genotypes identified in Israel, Brazil, USA, Nigeria and Spain. This report demonstrates the advantage of the broad range PCR technique for the diagnosis of culture-negative bacterial infections. We recommend that this technique should be performed routinely in veterinary laboratories, particularly in samples from CNS patients with CSF culture-negative results.

Abscesses caused by bacterial infections most commonly occur in exotic pets in Thailand including rabbit, hedgehog and sugar glider. Fastidious obligate anaerobic bacteria are mostly associated with abscesses in these animals. However, these bacteria are difficult to isolate from the abscesses and frequently excluded by the bias of conventional bacterial culture. This study performed a pilot investigation in clinical abscesses from 20 cases including 17 rabbits, 2 hedgehogs and 1 sugar glider using standard culture methods for both aerobes and anaerobes compared to a broad range nested-PCR targeting the 16S rRNA gene. The PCR detected bacterial DNA in 17 abscesses and sequencing of 10 PCR products revealed the close related to obligate anaerobes including *Bacteroides* spp. (2 rabbits, 1 hedgehog, 1 sugar glider), *Fusobacterium* spp. (4 rabbits, 1 hedgehog) and *Prevotella* spp. (1 rabbit). A housekeeping gene of these anaerobes, the *rpoB* gene was amplified from the 10 abscesses using a suicide PCR

protocol. Phylogenetic analysis of the *rpoB* gene demonstrated the species status of *Bacteroides thetaiotaomicron, Fusobacterium varium* and *F. nucleatum*. The *rpoB* gene amplification was failed for *Prevotella* spp. Five PCR positive samples were closely related to *Pseudomonas* spp. Phylogenetic analysis of the *rpoB* gene demonstrated *Pseudomonas auruginosa* in all 5 samples. Sequencing results of the 16S rRNA gene revealed that another two samples from rabbits were *Pasteurella* spp. and *E. coli*. Correlations between standard culture and PCR were found in *Pseudomonas auruginosa* from all 5 samples and 1 *Pasteurella* spp. Cultured-negative results occurred in 11 samples. Three of these cultured-negative samples were also negative for PCR detection. Standard culture isolated 2 *E. coli* and 1 β-*Streptococcus* that were discordance to PCR results. This broad range PCR was able to detect dominant anaerobic bacteria in abscesses that these dominant bacteria were overlooked by the conventional bacterial culture.

The eighty one anemic dogs with unknown causes were included in this study. The nested PCR detected 16S rDNA in 10 samples. Three types of organisms were detected in these blood samples including *Ehrlichia canis* (5), *Anapalsma platys* (3) and hemotropic *Mycoplasma* spp. (2). Co-infection with *E. canis* and *Mycoplasma hemocanis* was detected in two cases that presented with severe and life threatening clinical signs. Co-infection with multiple hemotropic pathogens could trigger more severity of hemolytic anemia in infected dogs. According to our knowledge, hemotropic *Mycoplasma* infections should be considered as a differential diagnosis in dogs with hemolytic anemia.

3. Objective

1. Develop molecular tools (broad range nested PCR) for detection of culture-negative bacteria in animal specimens (dogs and cats) including inflammatory tissue, abscess, urine, semen and cerebrospinal fluid samples
2. Develop molecular tools (Multilocus sequence typing; MLST for 2 housekeeping genes and Multispacer typing for 1 spacer region; MST) for both inter- and intra-species identifications of culture-negative bacteria
3. To characterize genetic diversity of the organisms detected in animal specimens

4. Research methodology

4.1 Analytical sensitivity and specificity

The analytical sensitivity of the nested broad-range PCR targeting the 16S rRNA gene was determined by testing of ten serial 10-fold dilutions which range from 10^{6}-10^{-3} bacterial colony forming unit (CFU/ml). The standard serial dilutions were prepared using stock culture of known bacterial culture that the known bacterial species performed for this process are listed in Table 1.

Table 1 Organisms used asses the sensitivity and specificity of PCR assays

Organism	Supplier
Escherichia coli	Bacteriology laboratory,
Staphylococcus species	Kasetsart Veterinary Teaching
Streptococcus species	Hospital
Pseudomonas species	
Klebsiella species	
Proteus species	

4.2 DNA extraction

DNA was extracted from each bacterial dilution (10^{6}-10^{-3} CFU/ml) using QIAamp ® DNA Mini Kits (QIAGEN Inc.) according to the manufacturer's instructions. Following extraction, DNA concentration and purity were measured by Nanodrop^{R} spectrophotometer.

4.3 Primers, PCR assays and DNA sequencing

PCR primers for the primary PCR were modified based on a previous report (Cai et al., 2013) and consisted of V1-F (5' AGAGTTTGATCCTGGCTCAG 3') and V9-R (5' GNTACCTTGT- TACGACTT 3'). PCR reactions for the primary PCR was performed using 1 µL of DNA in a 25 µL reaction containing 1 x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM of each primer and 0.04 U/µL Taq DNA polymerase (Invitrogen^{R}). The cycling conditions consisted of a pre-PCR step of 95°C for 5 minutes, followed by 40 cycles of 95°C for 60 seconds, 50°C for 60 seconds and an extension of 72°C for 90 seconds, with a final extension of 72°C for 10 minutes. These primary primers produced approximately 1,400 bp PCR products. PCR primers for the secondary PCR consisted of V3-F (5' ACTCCTACGGGAGGCAGCAG 3') and V6-R (5' CGACAGCCATGCANCACCT 3') that these primers were also modified as per a previous study (Lin et al., 2013). The PCR reaction for the secondary PCR were performed using 1 µL of DNA in a 25 µL reaction containing 1 x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM of each primer and 0.04 U/µL Taq DNA polymerase (Invitrogen^{R}). The cycling conditions consisted of a pre-PCR step of 95 °C for 5 minutes, followed by 45 cycles of 95°C for 60 seconds, 55°C for 45 seconds and an extension of 72°C for 45 seconds with a final extension of 72°C for 10 minutes. These secondary primers produced approximately 700 bp PCR products. All PCR products produced by secondary PCR were purified from agarose gel slices using an UltraClean^{TM} 15 DNA Purification Kit (MO BIO Laboratories Inc. West Carlsbad, California,

USA). Sequencing was performed using an ABI Prism™ Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) on an Applied Biosystems 3730 DNA Analyzer, following the manufacturer's instructions. The PCR primers used for MLST and MST were shown in Table 2.

Nucleotide sequences generated for all loci were analyzed using Chromas lite version 4.0 (http://www.technelysium.com.au) and aligned with reference sequences from GenBank using Clustal W (http://www.clustalw.genome.jp). Phylogenetic analysis of all loci was performed using distance method using Mega version 5.1 (Mega5: Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, Arizona, U.S.A.)

Table 2 PCR primers used for MLST and MST

Primer	Organism	Locus	Nucleotide sequences	Reference
Myco184-F1	*Mycoplasma*	16S rRNA	ACCAAGSCRATGATRGRTAGCTGG	This study
Myco1310-R1			ACRGGATTACTAGTGATTCCAACTTCAA	Cabello et al., 2013
Myco322-F2			GCCCATATTCCTACGGGAAGCAGCAGT	Varanut et al., 2011
Myco1310-R1			ACRGGATTACTAGTGATTCCAACTTCAA	Varanut et al., 2011
EC36-F1	*Ehrlichia canis*	*gp36*	GTATGTTTCTTTTATATCATGGC	Hsieh et al., 2010
EC36-R1			GGTTATATTTCAGTTATCAGAAG	
BF	*Bacteroides*	*rpoB*	CACTTGAGCAAYCGTCGTRT	Ko et al., 2007
BR			CCTTCAGGAGTYTCAATNGG	
Fn-RpoB-F1	*Fusobacterium*	*rpoB*	CTKGATGAAGAAACAGGAGART	Kim et al., 2010
Fn-RpoB-R1			AGTAGCAAGYGAYCCAATAAGT	
rpoB-PSF	*Pseudomonas*	*rpoB*	AGTTCATGGACCAGAACAACC	Sajben et al., 2011
rpoB-PTR			CCTTGACGGTGAACTCGTTC	

4.4 Sample collection

In this study, we used molecular diagnostic techniques to search for the organisms' DNA in the following samples held at Veterinary Teaching Hospitals, Kasetsart University that all samples were negative results from conventional bacterial culture methods. Total of 337 specimens were examined by the new broad range nested-PCR and a variety of clinical

specimens included urine, semen, abdominal effusion, pleural effusion, inflammatory tissue lesions and cerebrospinal fluid samples. Number of each sample tested in this study was presented in Table 3.

Table 3 Number of culture-negative samples examined by broad-range nested PCR

Clinical specimens	**No. of examination**
Urine	118
Semen	46
Peritoneal effusion	12
Pleural effusion	25
Tissue: abscess, mass,. wound, necrotic tissue, inflammatory tissue	65
Cerebrospinal fluid	71
Blood (Anemic patients)	81
Total	**418**

5. Result

5.1 Analytical sensitivity

The nested-PCR technique developed in this study was able to detect bacterial DNA which extracted from bacterial culture with the lowest concentration as 10^{-3} CFU/ml dilution. DNA concentration of all bacterial 10^{-3} CFU/ml dilutions were ranged from 0.5-1.9 ng/µl. Agarose gel electrophoresis results for each bacterial dilution were demonstrated in Fig. 1-6. All 700 bp-PCR products performed for these bacterial species were confirmed by sequencing and the sequencing results were identity to the referenced organisms as described previously.

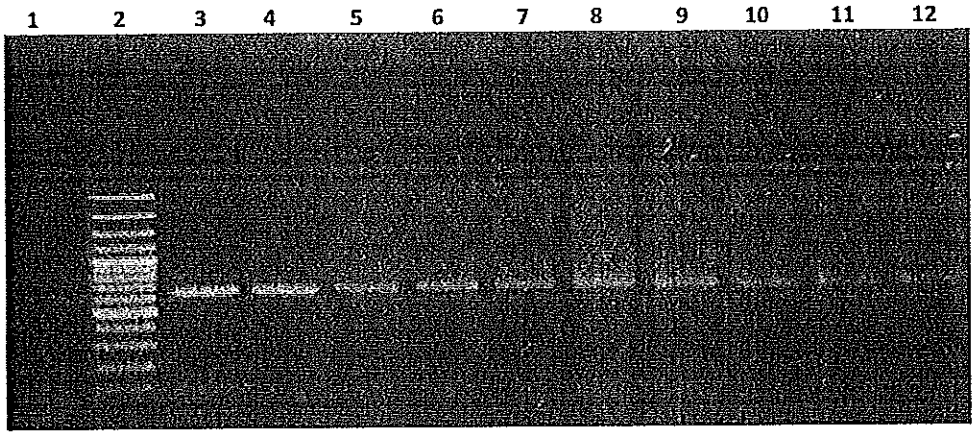

Figure 1 *Proteus* spp. agarose gel electrophoresis. Lane 1: negative control; Lane 2: 100 bp DNA marker; Lane 3-12: *Proteus* spp. in medium culture 10^{6}-10^{-3} CFU/ml, respectively.

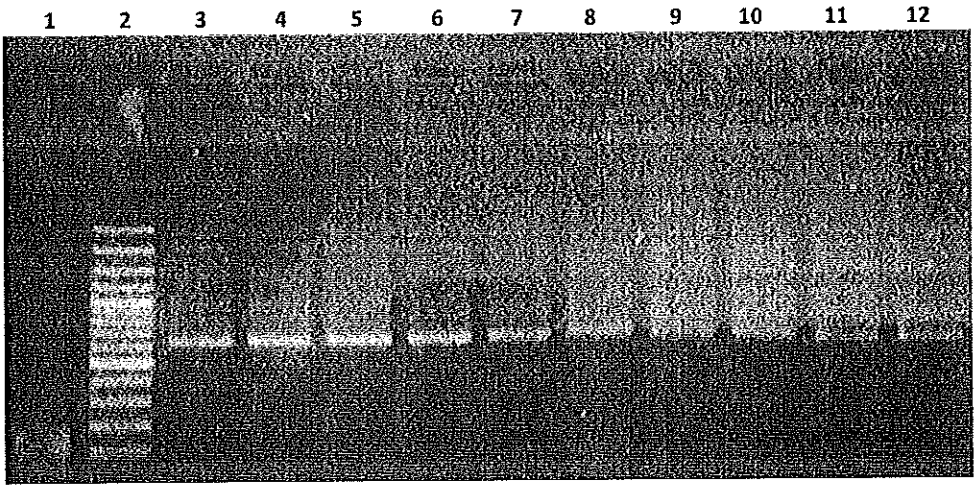

Figure 2 *Streptococcus* spp. agarose gel electrophoresis. Lane 1: negative control; Lane 2: 100 bp DNA marker; Lane 3-12: *Streptococcus* spp in medium culture 10^{6}-10^{-3} CFU/ml, respectively.

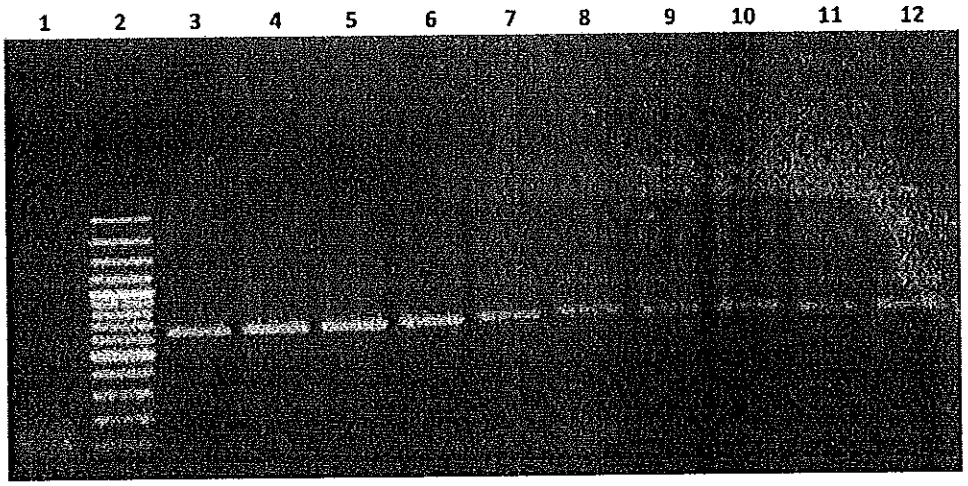

Figure 3 *Staphylococcus* spp. agarose gel electrophoresis. Lane 1: negative contròl; Lane 2: 100 bp DNA marker; Lane 3-12: *Staphylococcus* spp. in medium culture 10^{6}-10^{-3} CFU/ml, respectively.

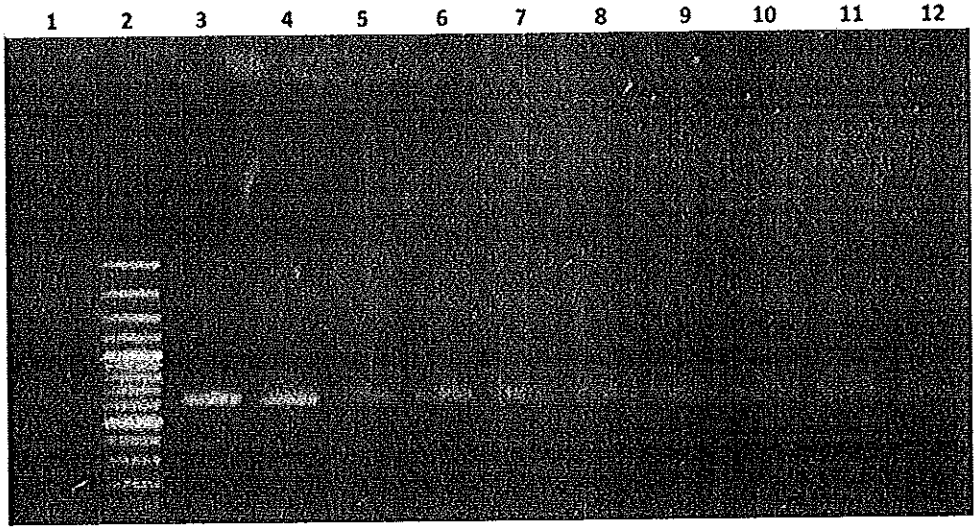

Figure 4 *Klebsiella* spp. agarose gel electrophoresis. Lane 1: negative control; Lane 2: 100 bp DNA marker; Lane 3-12: *Staphylococcus* spp. in medium culture 10^{6}-10^{-3} CFU/ml, respectively.

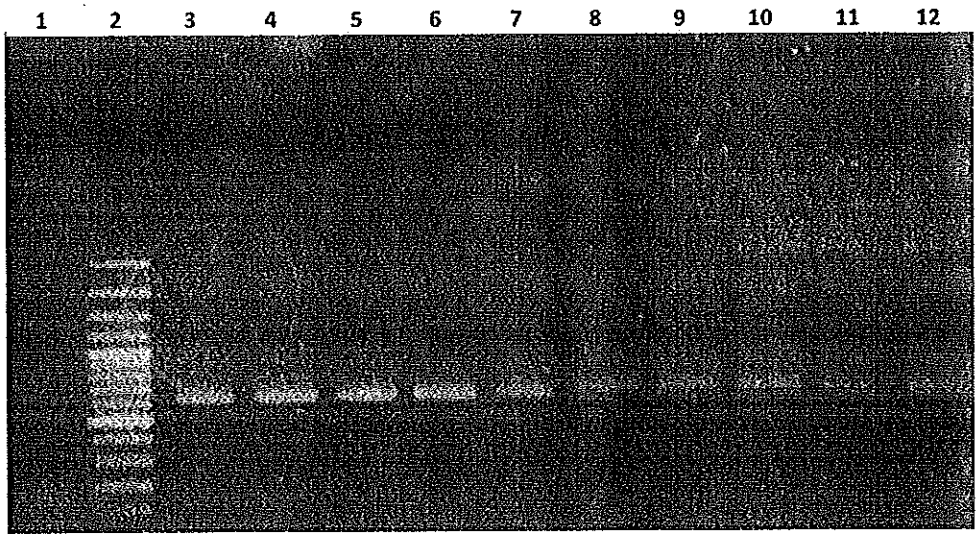

Figure 5 *E. coli* agarose gel electrophoresis. Lane 1: negative control; Lane 2: 100 bp DNA marker; Lane 3-12: *Staphylococcus* spp. in medium culture 10^{6}-10^{-3} CFU/ml, respectively.

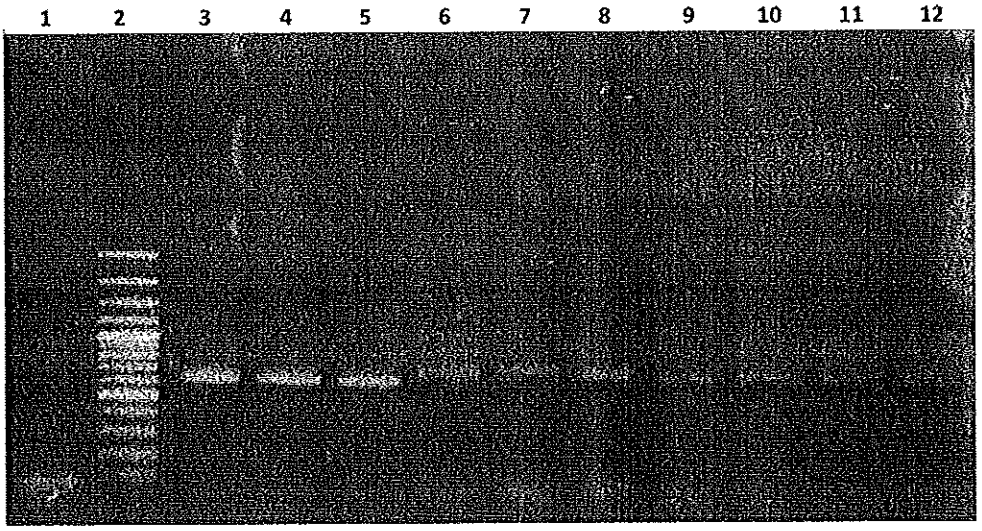

Figure 6 *Pseudomonas* spp. agarose gel electrophoresis. Lane 1: negative control; Lane 2: 100 bp DNA marker; Lane 3-12: *Staphylococcus* spp. in medium culture 10^{6}-10^{-3} CFU/ml, respectively.

5.2 Identification of bacterial infections in urogenital tract of dogs and cats pre-treated with antibiotics using broad-range nested PCR

The new nested PCR detected bacterial 16S rDNA in 25/46 semen specimens (54%) and 51/118 urine specimens (43%). The 118 urine samples were collected from 93 dogs and 25 cats and all semen samples were collected from 46 male dogs. All these specimens were submitted to the Bacteriology laboratory, Kasetsart Veterinary Teaching Hospital as part of the routine diagnosis. These cases were suspected as bacterial infections in their urogenital tract including lower urinary tract infection as cystitis and/or urethritis in both male and female of dogs and cats. Semen samples were submitted for the diagnosis of canine bacterial prostatitis. However, all these specimens were negative results from conventional bacterial culture methods. Bacterial 16S rDNA was detected in urine samples of 42/93 dogs (45%) and 9/25 cats (36%). In a total 42 positive urine samples in dogs, 35 samples were male dogs and 7 samples were female dogs. Seven positive urine samples were male cats and 2 samples were female cats. All DNA sequences derived from all positive PCR products were analyzed using the Basic Local Alignment Search Tool (BLAST) and the close related organisms compared to the GenBank database were demonstrated in Table 4-6

Table 4 Close related bacteria detected in urine samples from 42 dogs with urinary tract infections (UTI) seen at Veterinary Teaching Hospital, Kasetsart University in Bangkok

Close related organisms	**No of detections**
Burkholderia spp.	13
Staphylococcus spp.	4
Klebsiella spp.	2
Enterococcus spp.	4
Mycoplasma spp.	2
Ureaplasma spp.	1
Corynebacterium spp.	3
Macrococcus spp.	1
E. coli	5
Pseudomonas spp.	3
Comamonas spp.	1
Bacillus spp.	1
Sphingomonas spp.	1
Unidentified bacteria	1

Table 5 Close related bacteria detected in urine samples from 9 cats with urinary tract infections (UTI) seen at Veterinary Teaching Hospital, Kasetsart University in Bangkok

Close related organisms	**No of detections**
Pseudomonas spp.	1
Vagococcus spp.	1
Enterococcus spp.	1
Comamonas spp.	1
Enhydrobacter spp.	1
Acinetobacter spp.	1
Paenibacillus spp.	1
Staphylococcus spp.	1
Klebsiella	1

Table 6 Close related bacteria detected in semen samples from 25 dogs with urinary tract infections (UTI) and/or prostatitis seen at Veterinary Teaching Hospital, Kasetsart University in Bangkok

Close related organisms	No of detections
Stenotrophomonas spp.	1
Pseudomonas spp.	3
Enterobacter spp.	2
Burkholderia spp.	2
Cloacibacterium spp.	2
Enhydrobacter spp.	1
E. coli	2
Ureaplasma spp.	1
Citrobacter spp.	1
Staphylococcus spp.	1
Enterococcus spp.	1
Leptotrichia spp.	1
Moraxella spp.	1
Mycoplasma spp.	1
Actinobacillus spp.	1
Pasteurellaceae spp.	1
Chryseobacterium spp.	1
Acinetobacter spp.	1
Unidentified bacteria	1

5.3 Detection of pathogenic organisms in cerebrospinal fluid from uncommon cases with meningoencephalitis using broad range PCR

Case

A 10-year-old, 4.4 kg, intact female poodle presented with clinical signs of weakness in both hind limbs in May 2013 at the Veterinary Teaching Hospital, Kasetsart University, Bangkok, Thailand. The dog had a mild fever (39.5° C) and demonstrated hyperesthesia in the lumbar area. Radiographic imaging showed a normal picture of the spine and intervertebral disc spaces. A complete blood count was within reference ranges. An antipyretic drug, tolfenamic acid, was administered once by subcutaneous injection (4 mg/kg), combined with an analgesic drug, tramadol, administered orally (3 mg/kg). The mild fever improved within 24 hours but the dog still had signs of weakness in the hind limbs and hyperesthesia in the lumbar area. Intervertebral disc disease at the lumbo-sacral area was initially diagnosed and anti-inflammatory doses of corticosteroid were applied for 7 days, after which the clinical signs of weakness in both hind limbs had slightly improved. A month after treatment, in June 2013, there were no signs of hind limb weakness however, recurrence of the same clinical signs occurred for 3 months from June to August 2013, and intermittent courses of 7-day duration corticosteroid were prescribed at subsequent monthly checkups. On 10th September 2013, the dog was hospitalized with clinical signs of hyperesthesia along the vertebral column and neurological deficits, including disoriented mental status, unilateral palpebral reflex deficit and vestibular and cerebellar ataxia. These findings suggested multifocal brain lesions with progressive loss of neurological functions. A blood test revealed leukocytosis (26,000 cells/µl) with left shift and mild anemia (HCT 26.8%). Platelet numbers and other blood chemistry profiles were normal. Infectious encephalitis was suspected as a result of the clinical signs and leukocytosis. A sample of CSF was submitted to

the diagnostic laboratory for standard bacterial culture and cytological examination. The result of CSF culture for bacteria using standard techniques was negative. Subsequently, a broad-range nested PCR targeting the 16S rRNA gene was performed to confirm bacterial meningoencephalitis. The dog was treated with amoxicillin/clavulanic acid (30 mg/kg), dexamethasone (0.05 mg/kg), dimenhydrinate (8 mg/kg), and vitamin B.

The 16S rDNA sequence was analyzed using the Basic Local Alignment Search Tool (BLAST). Bacterial 16S rDNA was detected in the CSF by PCR and the DNA sequence derived from the PCR product was most closely related to *Ehrlichia canis* in the GenBank database; accession number KC479024, with 99% identity.

Cerebrospinal fluid analysis demonstrated an elevated protein level (197 mg/dl, reference range 0-30 mg/dl) and mixed cell pleocytosis (34/µl, reference range 0-5/µl). Cytological findings included non-degenerative neutrophils, small lymphocytes, mononuclear cells and macrophages with engulfed red cells and/or fat droplets. In addition, intracytoplasmic inclusion bodies, consistent with *Ehrlichia* spp. morulae, were found in mononuclear cells. These findings indicated possible meningoencephalitis caused by *E. canis* (Fig. 7).

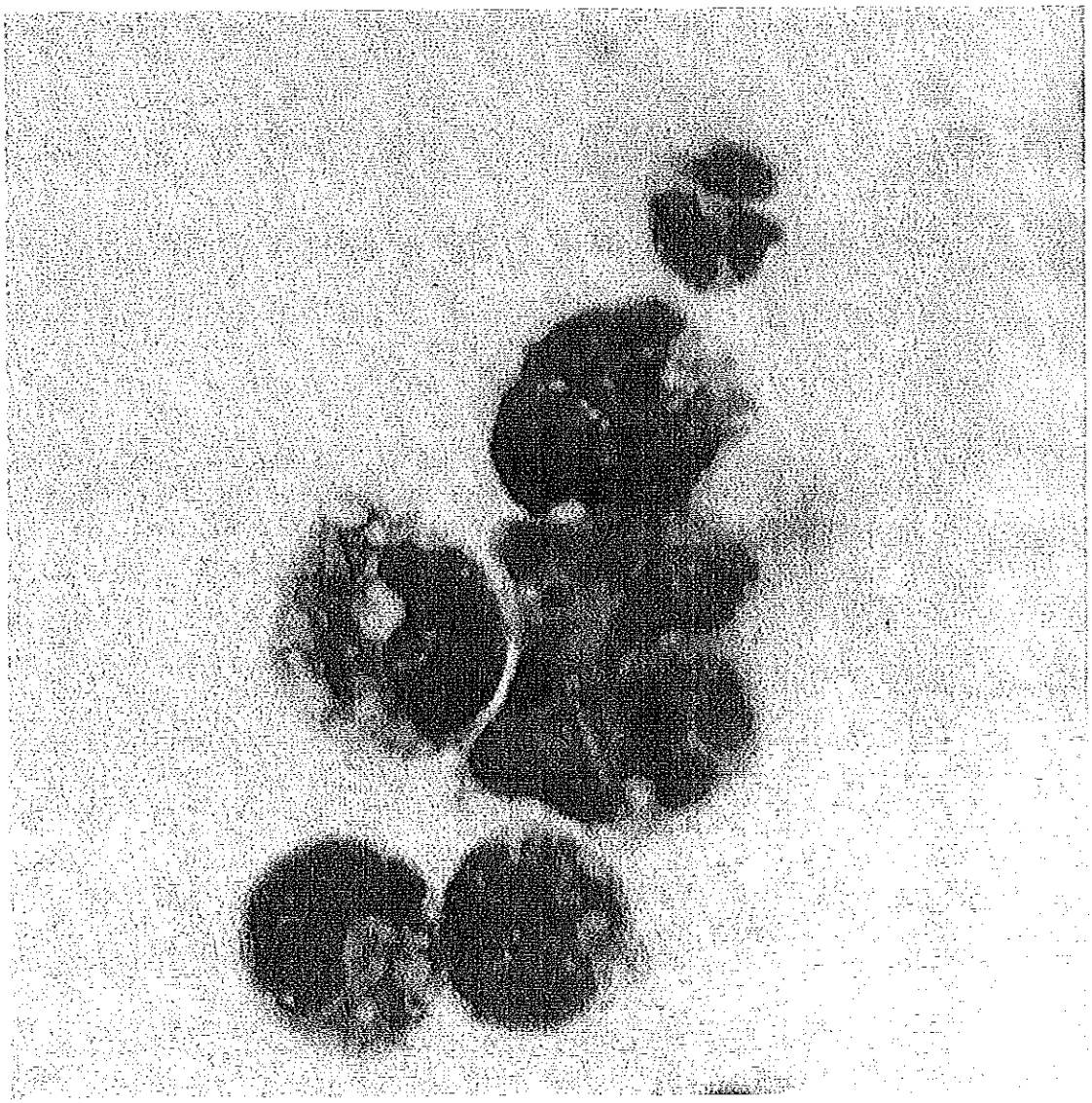

Figure 7 Photomicrograph of cerebrospinal fluid cytology. Mixed cell pleocytosiscomposed of mononuclear cells, lymphocytes and neutrophil. Several round-shaped basophilic intracytoplasmic inclusions were seen in mononuclear cells. (Wright-Giema stain).

Following the infrequent findings from the BLAST algorithm and CSF analysis, a suicide PCR protocol was adopted to confirm the organism's genetic identity and to prevent cross contamination of 16S rDNA amplicons (Raoult et al., 2000). The suicide PCR was adopted in order to confirm the unexpected finding of *E. canis* DNA in the CSF of this unusual clinical case and because cross contamination of the 16S rRNA amplicons could have occurred. The amplification of a novel gene, the *gp36* of *E. canis* was performed once without the addition of a positive control. This novel locus had never been amplified previously and was targeted only once with fresh primers. The suicide PCR protocol confirmed that detection using the 16S PCR had not resulted from cross contamination in our laboratory.

The PCR targeting the *gp36* locus of *E. canis* was performed and DNA sequencing of the resulting PCR product was completed after the first amplification. PCR primers for the *gp36* gene, EC36-F1 (5'-GTATGTTTCTTTTATATCATGGC-3') and EC36-R1 (5'-GGTTATATTT-CAGTTATCAGAAG-3') were used as per a previous study (Hsieh et al., 2010).

The 16S rRNA and *gp36* loci sequences were submitted to GenBank with accession numbers KM879929 and KM879930, respectively. A Neighbor-Joining phylogenetic tree of the *gp36* gene was constructed and revealed that the *E. canis*-like genotype detected in the present study was closely related to other validated genotypes reported from Israel, Brazil, USA, Spain and Nigeria (89.5-96.1% identity) (Fig. 8). These genotypes were classified as *E. canis* cluster A in a previous report (Zweygarth et al., 2014). However, the dog died before advanced brain imaging was performed. The molecular diagnosis and CSF cytology results were recorded subsequently.

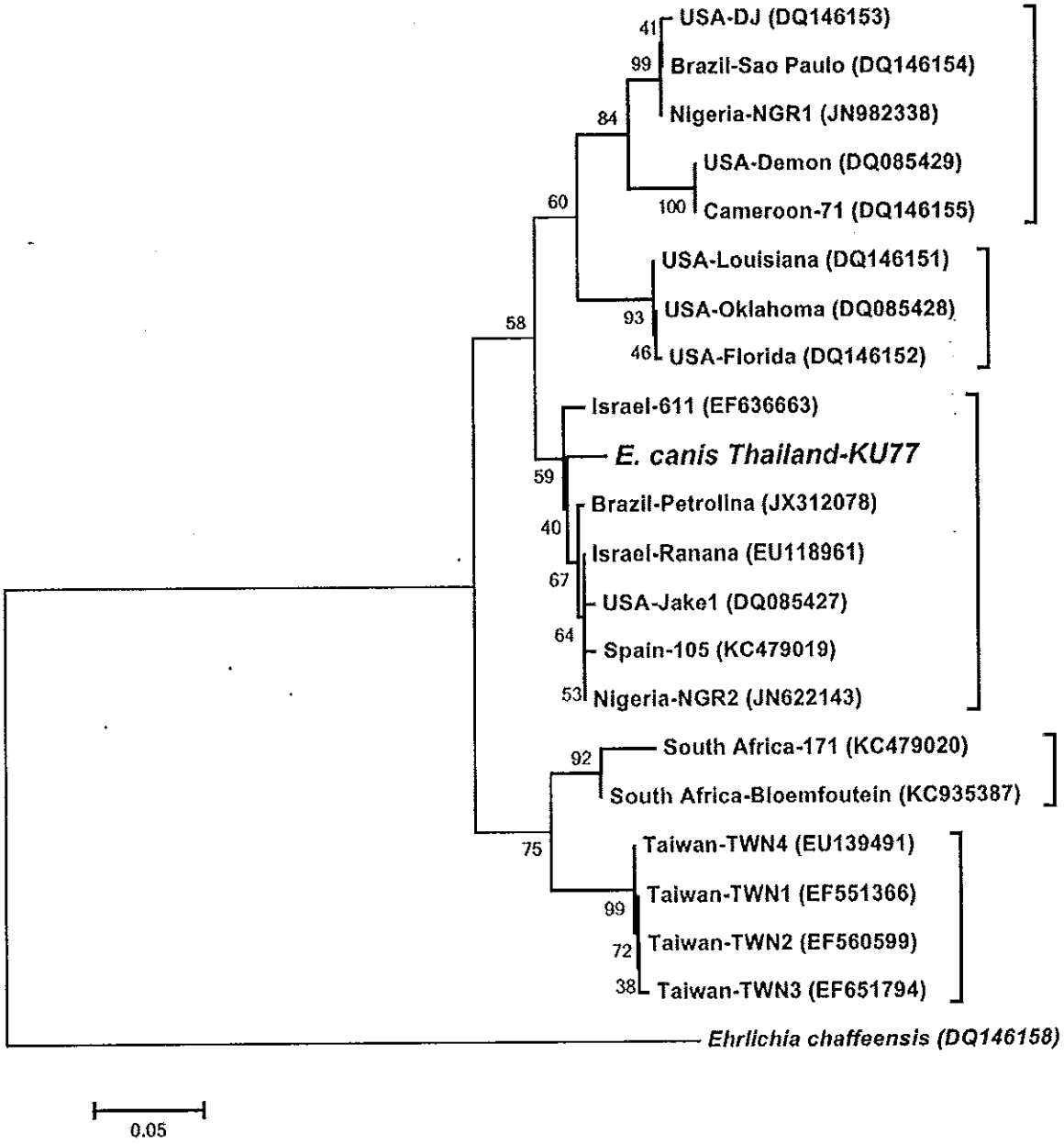

Figure 8 Neighbor-Joining phylogenetic tree of the *gp36* gene of *Ehrlichia canis* detected in the CSF of a dog in Thailand and validated genotypes of *Ehrlichia canis*. Percentage bootstrap support (>40%) from 1000 pseudoreplicates is indicated at the left of the supported node.

Ehrlichia canis, a tick-borne pathogen, mainly transmitted by *Rhipicephalus sanguineus,* is the cause of canine monocytic ehrlichiosis (CME), which is a multisystemic disease resulting in hematological abnormalities and respiratory, ocular or neurological sequelae (Baba et al., 2012). Coagulation disorder, due to severe thrombocytopenia and platelet dysfunction, is the most prominent sign of infection with *E. canis* (Bulla et al., 2004, de Castro et al., 2004). The organism has resulted in high morbidity and mortality in dogs in Southeast Asia since it was first identified in military dogs during the Vietnam War in the late 1980s (Greene, 2012). However, published reports in this region, of both clinical manifestations and genetic studies, are very limited (Foongladda et al., 2011, Jirapattharasate et al., 2012, Pinyoowong et al., 2008).

This dog showed typical clinical symptoms of bacterial meningoencephalitis as described. Therefore, the initial treatment was based on a diagnosis of bacterial encephalitis. Unfortunately, as a result of the unusual clinical and hematological findings of this case, *E. canis* was not included in the differential diagnosis and thus initially there were no specific tests used for *E. canis* infection, for example specific PCR or antibody detection. In retrospect, the rapidly progressing CNS signs in this dog most likely resulted from *E. canis* infection, confirmed by the CSF cytology, sequencing result of the 16S rRNA gene and phylogenetic analysis of the *gp36* gene. Neurological deficits of ehrlichial meningoencephalitis are influenced by plasma cell infiltration of the meninges or hemorrhage in the cerebral or spinal cord parenchyma (Baba et al., 2012, Woody et al., 1991). The first detection of *E. canis* in CSF was reported from the US in 1989 and the report described seizures as the dominant sign in the infected dog, together with non-regenerative anemia and chronic thrombocytopenia (Meinkoth et al., 1989). A subsequent report in 2012 from Japan also described ataxia of the hind limbs in the infected dog, with non-regenerative anemia and severe thrombocytopenia (Baba et al., 2012). In addition, the case from

Japan reported xanthochromia in the CSF, which is consistent with subarachnoid hemorrhage. There is less information in the literature describing meningoencephalitis associated with ehrlichiosis in dogs without thrombocytopenia and bleeding tendency, as was the case in our patient. A normal platelet count and transient thrombocytopenia were previously reported from a dog with an uncommon case of severe hepatitis associated with acute *E. canis* infection (Mylonakis et al., 2010). The use of ampicillin and enrofloxacin to treat this dog was based on an initial diagnosis of infectious hepatitis caused by leptospirosis. *Ehrlichia* spp. morulae were subsequently found following liver impression cytology and the treatment was changed to doxycycline at 7 days post admission (Mylonakis et al., 2010).

The CSF abnormalities reported in this case are non-specific and could easily be attributed to general bacterial meningoencephalitis. Presumptive diagnosis and treatment in such cases is frequently performed for bacterial or other causes of CSF pleocytosis, and canine erhlichiosis is regularly excluded from the differential diagnosis, particularly in non-thrombocytopenic cases. Consequently in this case, there was no administration of doxycycline, which is the drug of choice for CME. Although various pathogens could have been ruled out using specific PCR assays for each organism, the broad range PCR technique we adapted was quicker and more advantageous and should be recommended for the diagnosis of CNS patients with CSF culture negative results, including the diagnosis of atypical CME.

5.4 Identification of uncultured-bacteria detected from abscesses of pet animals by broad range nested 16S rRNA PCR

This part performed a pilot investigation in clinical abscesses from 20 cases including 17 rabbits, 2 hedgehogs and 1 sugar glider using standard culture methods for both aerobes and anaerobes compared to a broad range nested-PCR targeting the 16S rRNA gene. The PCR detected bacterial DNA in 17 abscesses and sequencing of 10 PCR products revealed the close related to obligate anaerobes including *Bacteroides* spp. (2 rabbits, 1 hedgehog, 1 sugar glider), *Fusobacterium* spp. (4 rabbits, 1 hedgehog) and *Prevotella* spp. (1 rabbit). A housekeeping gene of these anaerobes, the *rpoB* gene was amplified from the 10 abscesses using a suicide PCR protocol. Phylogenetic analysis of the *rpoB* gene demonstrated the species status of *Bacteroides thetaiotaomicron*, *Fusobacterium varium* and *F. nucleatum*. The *rpoB* gene amplification was failed for *Prevotella* spp. Five PCR positive samples were closely related to *Pseudomonas* spp. Phylogenetic analysis of the *rpoB* gene demonstrated *Pseudomonas auruginosa* in all 5 samples. Sequencing results of the 16S rRNA gene revealed that another two samples from rabbits were *Pasteurella* spp. and *E. coli*. Correlations between standard culture and PCR were found in *Pseudomonas auruginosa* from all 5 samples and 1 *Pasteurella* spp. Cultured-negative results occurred in 11 samples. Three of these cultured-negative samples were also negative for PCR detection. Standard culture isolated 2 *E. coli* and 1 β-*Streptococcus* that were discordance to PCR results. This broad range PCR was able to detect dominant anaerobic bacteria in abscesses that these dominant bacteria were overlooked by the conventional bacterial culture.

We could classify our results from 20 cases into 4 groups including results in agreement between conventional culture and PCR, negative results for both techniques, no growth by standard culture but found anaerobes and discordant results as presented in Figure 9.

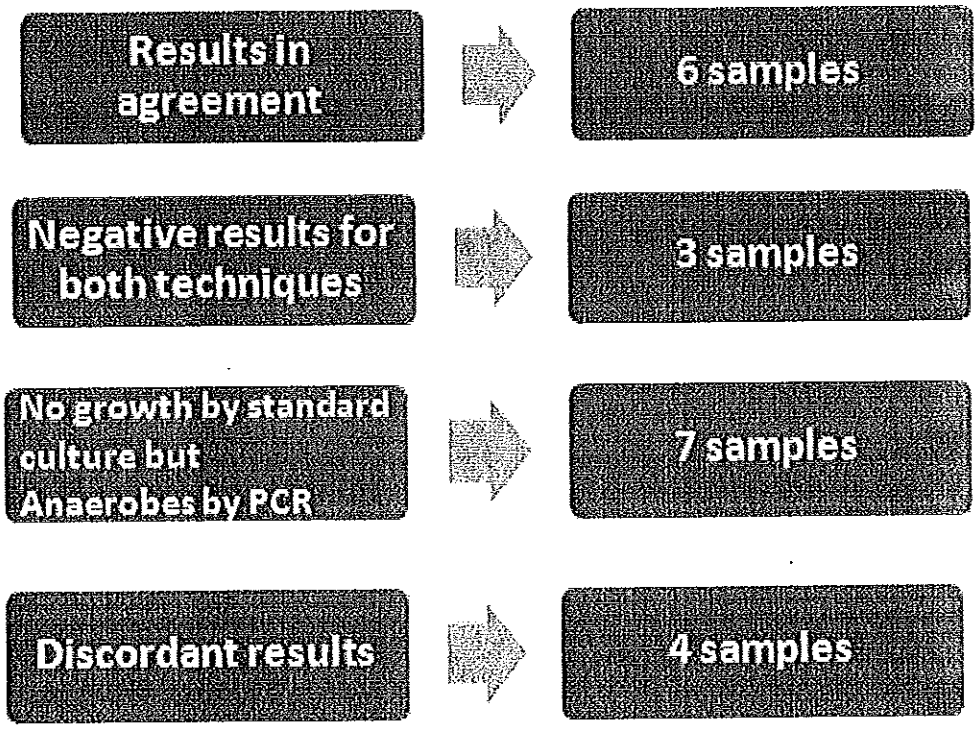

Figure 9 Classification of the PCR results compared with conventional culture results from 20 cases of abscesses

Table 7 Agreement results

Specimen	Patient	Standard culture	DNA sequencing	Clinical data
Abscess in abdominal cavity	Rabbit	*Pseudomonas* sp.	*Pseudomonas aeruginosa*	No recurrent after removed
Left tarsal joint abscess	Rabbit	*Pseudomonas* spp.	*Pseudomonas aeruginosa*	No recurrent after removed
Abscess at left lower eye lid	Rabbit	*Pseudomonas* spp.	*Pseudomonas aeruginosa*	Recurrent multiple sites of abscesses
Abscess at left ear pinna	Rabbit	*Pseudomonas* spp.	*Pseudomonas aeruginosa*	No follow up data
Tooth root abscess	Rabbit	*Pasteurella multocida*	*Pasteurella multocida*	No recurrent after removed
Tooth root abscess	Rabbit	*Pseudomonas* spp.	*Pseudomonas aeruginosa*	Chronic infection with antibiotic resistant

Pseudomonas is one of most common bacteria causing abscesses in rabbits and in our investigation. We found the same results between PCR and conventional culture methods (Table 7). From genetic analysis using the *rpoB* gene, we confirmed the species status of *Pseudomonas aeruginosa* in 5 samples from a total of 20 samples. One sample was *Pasteurella multocida.* A Neighbor-Joining phylogenetic tree of the *rpoB* gene was constructed and revealed that the genotype of *Pseudomonas aeruginosa* in 5 samples detected in the present study were closely related to other validated genotypes reported from GenBank (98.5-99.1% identity) (Fig. 10).

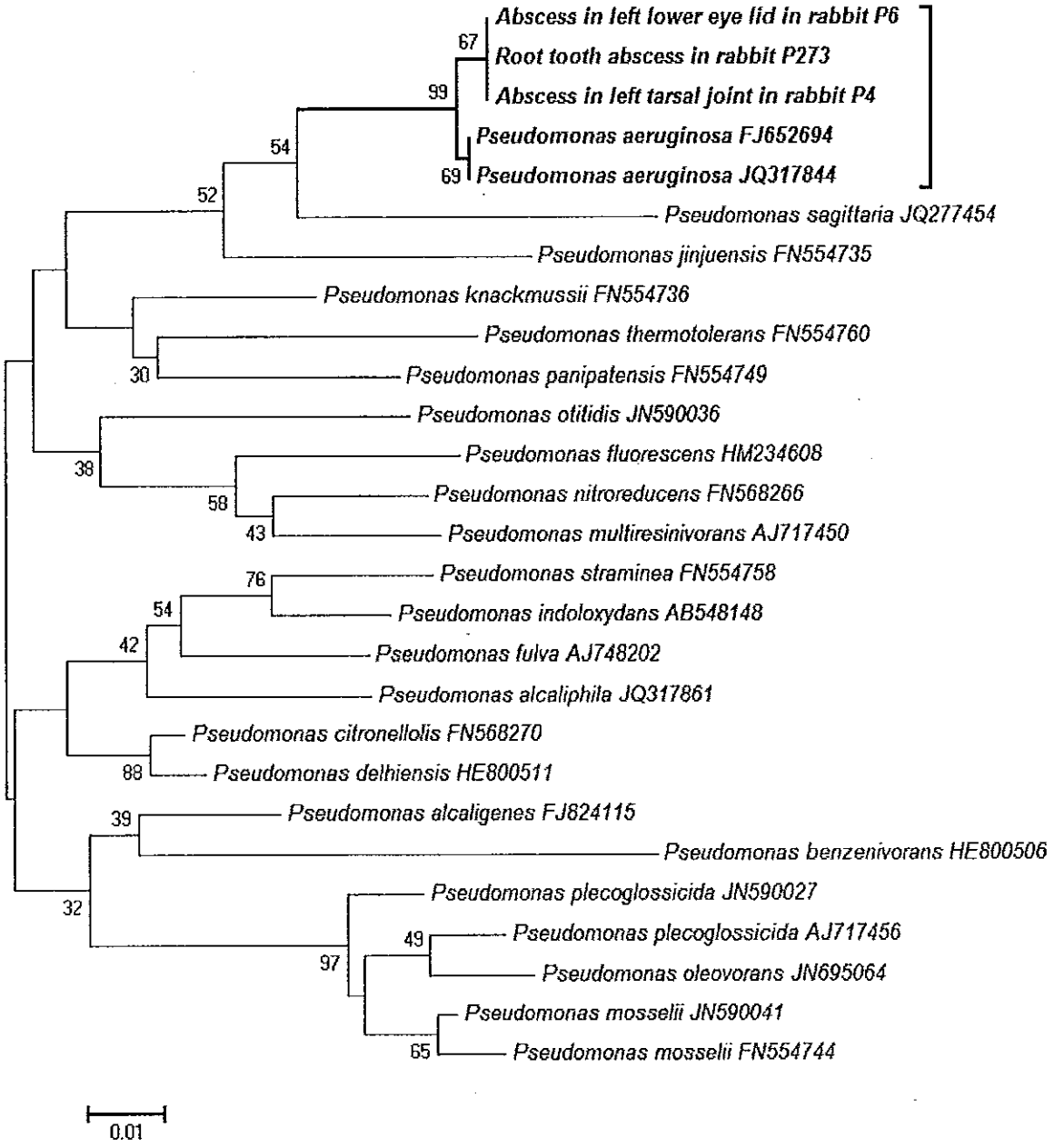

Figure 10 Neighbor-Joining phylogenetic tree of the *rpoB* gene of *Pseudomonas* species detected in the abscesses and validated genotypes of *Pseudomonas* species.

Table 8 Both negative results

Specimen	Patient	Standard culture	DNA sequencing	Clinical data
Tooth root abscess	Rabbit	No growth	PCR negative	No recurrent after removed
Abscess at interdigital area	Rabbit	No growth	PCR negative	Hind limb amputation
Left tarsal joint abscess	Rabbit	No growth	PCR negative	No recurrent after removed

Negative results were in agreement in both techniques. These negative results could confirm that these cases may not be caused by bacterial infections (Table 8). In all 20 cases, PCR can detect bacterial DNA in 17 cases but the PCR found negative results in 3 cases as shown in Table 8. However, negative results occurred in 11 samples using a conventional bacterial culture technique and all these cultured-negative samples were found anaerobic bacterial DNA in 7 samples (Table 9). Anaerobes in these cases were mostly originated from periodontal bacteria that were related to the site of infections (Table 9). Most cases were tooth root abscesses. Our new broad range PCR demonstrated the benefit as an alternative tool which can help to recheck the result of conventional culture, particularly anaerobic bacteria culture. Maximum-likelihood and Neighbor-Joining phylogenetic trees of the *rpoB* gene of anaerobes detected in this study were constructed to confirm the species of these bacteria including *Fusobacterium* spp. and *Bacteroides* spp. (Fig 11, 12). Aerobic bacteria were isolated including *β-Streptococcus* sp. and *E. coli* using the conventional bacteria culture (Table 10). However, the nested-PCR detected the dominant bacteria in these specimens that were discordance compared to the results from conventional culture.

Table 9 No growth by standard culture but anaerobes by PCR

Specimen	Patient	Standard culture	DNA sequencing	Clinical data
Tooth root abscess	Sugar glider	No growth	*Bacteroides fragilis*	Recurrent after surgical treatment
Tooth root abscess	Rabbit	No growth	*Bacteroides fragilis*	Hemi-mandibulectomy
Abscess in abdominal cavity	Hedgehog	No growth	*Fusobacterium varium*	Died after peritoneal drainage
Tooth root abscess	Rabbit	No growth	*Fusobacterium varium*	Recurrent multiple sites of abscesses
Tooth root abscess	Rabbit	No growth	*Prevotella* spp.	Recurrent multiple sites of abscesses
Subcutaneous abscess	Rabbit	No growth	*Fusobacterium varium*	No recurrent after removed
Tooth root abscess	Rabbit	No growth	*Fusobacterium nucleatum*	Chronic infection progressed to systemic signs

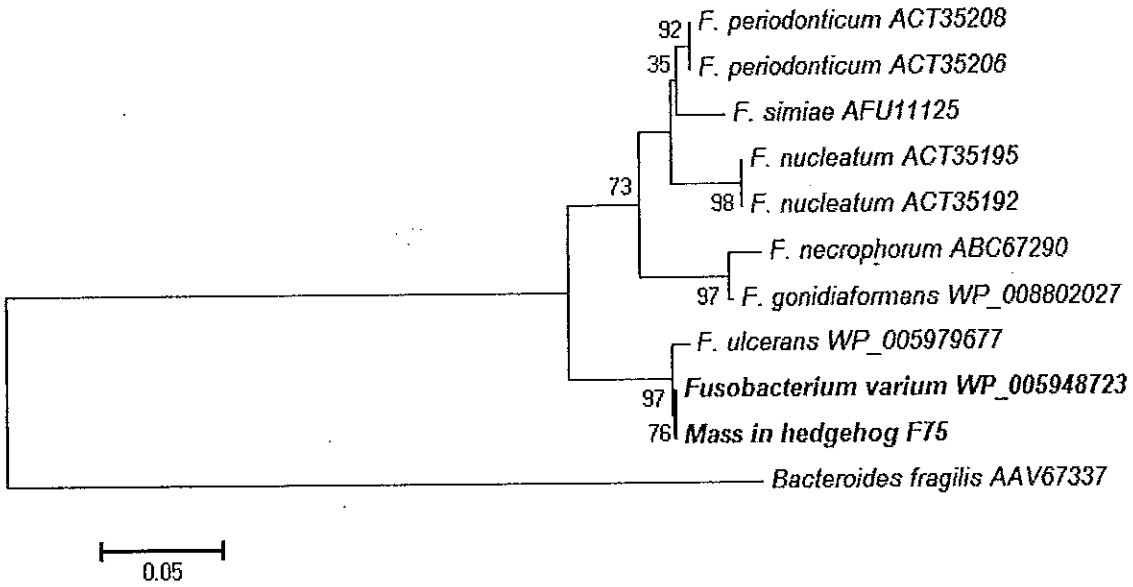

Figure 11 A Maximum-likelihood tree of the rpoB protein (RNA polymerase beta subunit) sequences from *Fusobacterium* spp. Percentage bootstrap support from 1000 pseudo-replicates is indicated at the left of the supported node.

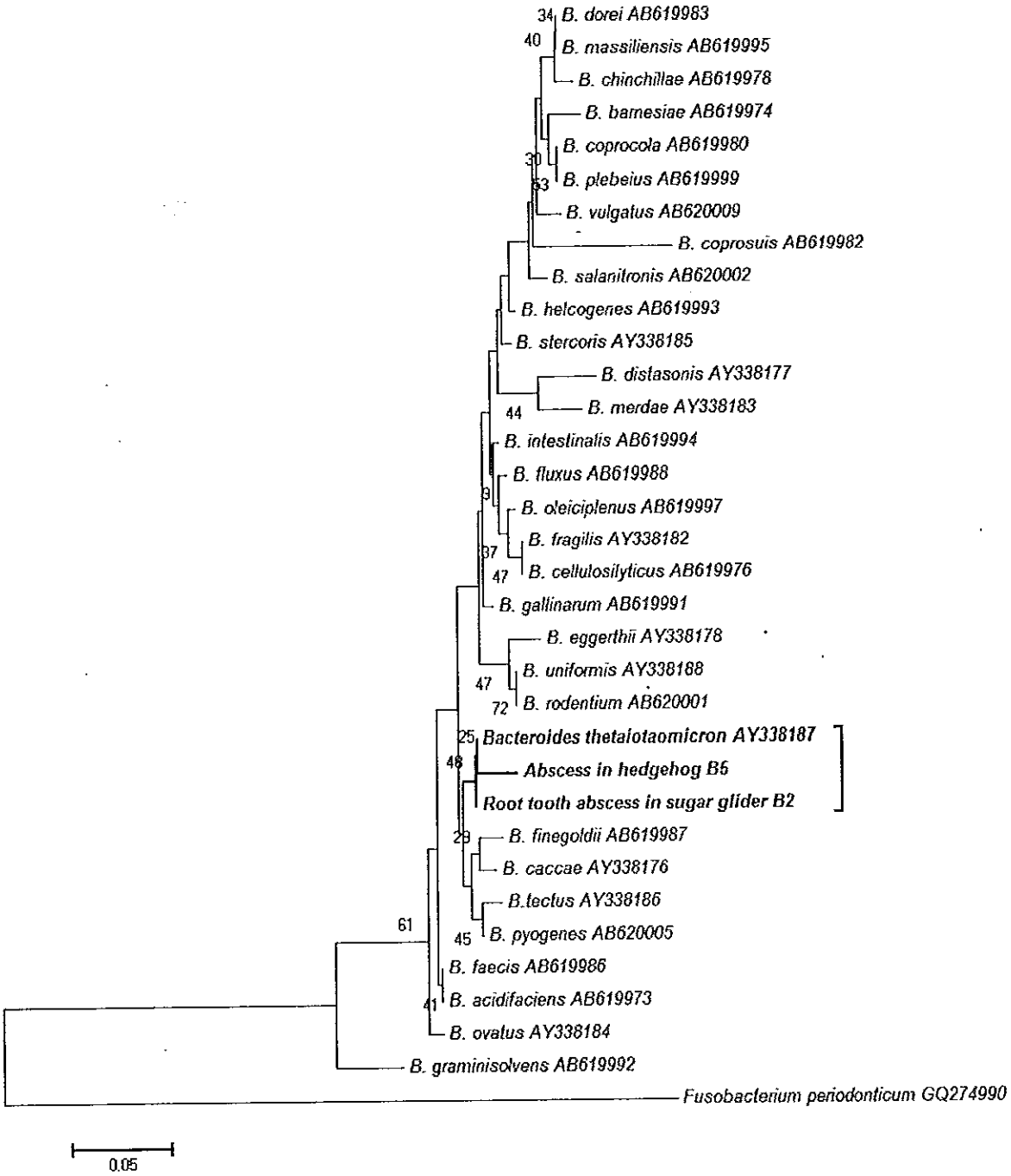

Figure 12 Neighbor-Joining phylogenetic tree of the *rpoB* gene of *Bacteroides* spp. detected in the abscesses and validated genotypes of *Bacteroides* spp. Percentage bootstrap support from 1000 pseudoreplicates is indicated at the left of the supported node.

Table 10 Discordant results

Specimen	Patient	Standard culture	DNA sequencing	Clinical data
Subcutaneous abscess	Hedgehog	*β-Streptococcus* sp.	*Bacteroides fragilis*	Mast cell tumor
Tooth root abscess	Rabbit	*E. coli*	*Fusobacterium nucleatum*	Recurrent multiple sites of abscesses
Retrobulba abscess	Rabbit	*E. coli*	*Bacteroides massiliensis*	Recurrent same site of the abscess
Tooth root abscess	Rabbit	No growth	*E. coli*	Recurrent multiple sites of abscesses

5.5 Identification of uncultured-bacteria detected in anemic dogs by broad range nested 16S rRNA PCR

Eighty one blood samples collected from anemic dogs were previously screened for *Babesia canis* infections. This organism is one of most important for intraerythrocytic protozoan in dogs that is transmitted by ticks. A variety of hemotropic bacteria causing anemia in dogs has been reported for example *Anaplasma* spp., *Ehrlichia* spp. and canine hemotropic *Mycoplasma* spp. Specific antibiotics for these organisms are recommended for the treatment. However, the hemoculture protocols for the organisms have not been reported yet and these difficulties have made the complications for the diagnosis of these infections in pet dogs. The nested PCR detected bacterial 16S rDNA in 10/81 blood specimens (12.34%). The BLAST results revealed three types of organisms detected in these blood samples including *Ehrlichia canis* (5), *Anapalsma platys* (3) and hemotropic *Mycoplasma* spp. (2). The specific primers for hemotropic *Mycoplasma* spp. were also tested in these blood specimens and found the associations between these two sets of primers. Co-infection with *E. canis* and *Mycoplasma hemocanis* was detected in two cases. These two male dogs presented with jaundice, severe anemia, severe thrombocytopenia, leukocytosis, mild azotemia and hepatitis. Hemolytic anemia was noted in these cases. Correlations between *Mycoplasma hemocanis* and anemia in dogs are less significant. However, co-infection with other hemotropic pathogens could trigger more severity of hemolytic anemia in infected dogs. According to our knowledge, hemotropic *Mycoplasma* infections should be considered as a differential diagnosis in dogs with hemolytic anemia.

The phylogentic tree of the 16S rRNA gene for *Mycoplasma* spp. was constructed to confirm the genetic status of *Mycoplasma* spp. detected in blood of anemic dogs (Fig. 13). *Candidatus* Mycoplasma hematoparvum was detected in an anemic dog in this study that this

Mycoplasma sp. has been reported as a cause of disease in veterinarians (Maggi et al., 2013a, 2013b). This was the first report of canine hemoplasma infections in Thailand. Major arthropod vectors for this *Mycoplasma* sp. in dogs have not been identified yet. Furthermore, investigations of these hemotropic organisms should be performed in arthropod vectors including ticks, fleas, biting flies and mosquitoes in Thailand.

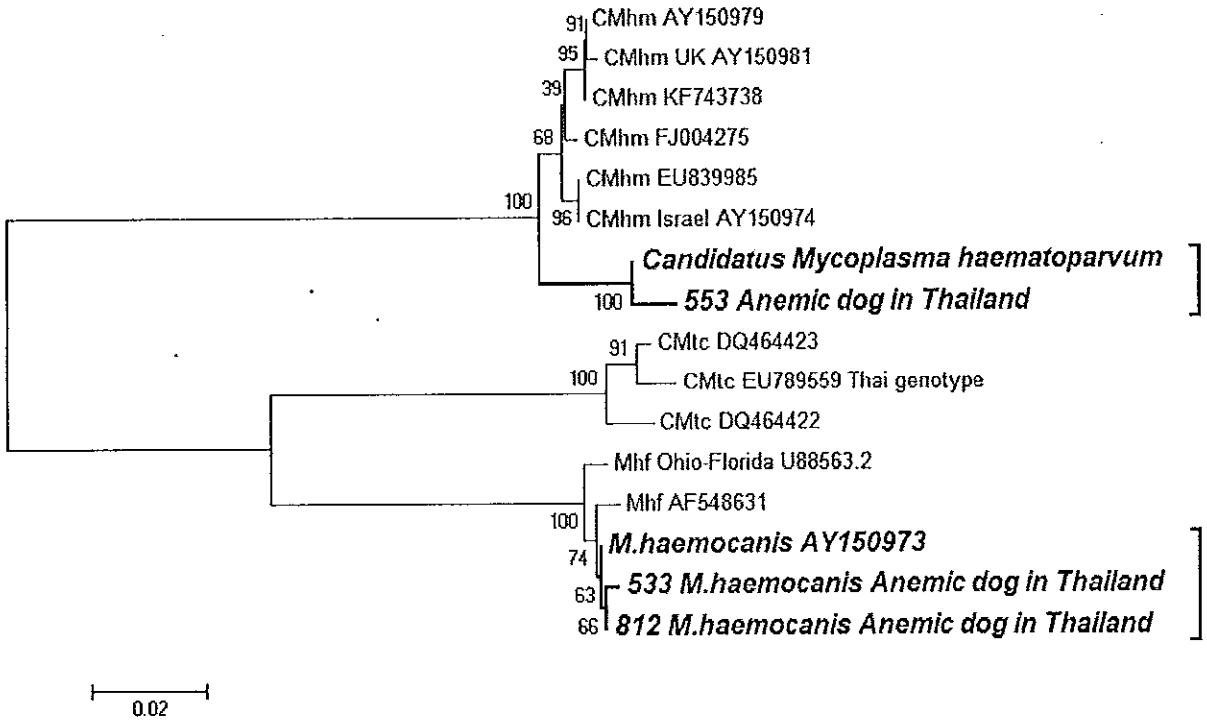

Figure 13 Neighbor-Joining phylogenetic tree of the 16S rRNA gene of hemotropic *Mycoplasma* spp. detected in the anemic dogs. Percentage bootstrap support from 1000 pseudoreplicates is indicated at the left of the supported node.

6. Conclusion and Discussion

The broad range nested-PCR developed in this study presented high sensitivity for the detection of various bacteria species. DNA concentrations were also very low in the lowest bacterial dilutions. However, the correlations between DNA concentration and each bacterial dilution should be performed using statistical analysis in further processes. The concept of the broad-range PCR reported previously has been proved that these tools are actually useful for diagnosis of culture-negative bacterial infections in a variety of clinical specimens (Rampini et al., 2011, Levy et al., 2012, Bosshard et al., 2003). Previous studies of these broad-range PCRs also revealed the huge advantage compared with the conventional bacterial culture techniques. Currently, these tools have been accepted for the applications in culture-negative bacteria in human medicine. However, the bias detections of theses PCRs has been considered that most universal bacterial primers designed in many reports were not able to cover all species of bacteria (Dan-Ping Mao et al., 2012). The coverage evaluation of these 16S rRNA gene primers was proposed (Dan-Ping Mao et al., 2012) and conservative fragments of this gene in the diversity of bacteria were also reported in order to increase the coverage rate of novel primers' designs (Wang et al., 2009). This study created a novel nested-PCR that the primers were modified from the report of conservative fragments in a previous study (Cai et al., 2013). In addition, this study also developed more sensitivity detection using two sets of primers to be a nested-PCR. However, the high sensitivity detection created by this tool could be disadvantage particularly, in cross contaminations by PCR amplicons.

Although this PCR detection will be a useful tool for the diagnosis of culture-negative bacterial infections, antibiotic sensitivity tests are still needed using the conventional bacterial culture techniques which could not be replaced by this PCR detection. The results of this broad-

range PCR detection revealed more novel pathogenic bacteria in a variety of clinical specimens in animals that are very important for the improvement of specific culture techniques which require special culture media (solid, semisolid and solution media) and special culture conditions.

These results also confirmed some errors of anaerobe culture in the routine laboratory and the nested-PCR detected the dominant bacteria in these specimens that were discordance compared to the results from conventional culture. According to the results of anaerobic bacteria in this pilot study, infection in these pets caused by anaerobes may be underestimated and could affect to the low successful rate of treatment in those cases based on the results from conventional culture. Finally, we have to improve our protocol for both transport and culture of anaerobic bacteria. The specific antibiotic bead for anaerobic bacteria is our next target to improve the treatment for those abscesses. We could implant the beads to the surgical area after removed the abscess.

The detection of bacterial 16S rDNA in various clinical specimens suggests the advantage of the broad range PCR technique, developed in this study, for the diagnosis of culture-negative bacterial infections in pet animals. In conclusion, this broad range PCR technique should be recommended in veterinary laboratories, particularly in inflammatory samples with culture-negative results.

References

Baba K, Itamoto K, Amimoto A, et al. *Ehrlichia canis* infection in two dogs that emigrated from endemic areas. J Vet Med Sci 2012;74:775-778.

Bosshard, P.P., Abels, S., Zbinden, R., Bottger, E.C., Altwegg, M.,. Ribosomal DNA sequencing for identification of aerobic gram-positive rods in the clinical laboratory (an 18-month evaluation). J Clin Microbiol 2003;41, 4134-4140.

Bulla C, Kiomi Takahira R, Pessoa Araujo J Jr, et al. The relationship between the degree of thrombocytopenia and infection with *Ehrlichia canis* in an endemic area. Vet Res 2004;35:141-146.

Cai L, Ye L, Tong AH, et al. Biased diversity metrics revealed by bacterial 16S pyrotags derived from different primer sets. PloS One 2013;8:e53649.

de Castro MB, Machado RZ, de Aquino LP, et al., Experimental acute canine monocytic ehrlichiosis: clinicopathological and immunopathological findings. Vet Parasitol 2004;119:73-86.

Drancourt, M., Bollet, C., Carlioz, A., Martelin, R., Gayral, J.P., Raoult, D. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J Clin Microbiol 2000;38, 3623-3630.

Foongladda S, Inthawong D, Kositanont U, Gaywee J. *Rickettsia*, *Ehrlichia*, *Anaplasma*, and *Bartonella* in ticks and fleas from dogs and cats in Bangkok. Vector Borne Zoonotic Dis 2011;11:1335-1341.

Greene CE:, *Ehrlichia* and *Anaplasma* infections. *In*: Infectious diseases of the dog and cat 2012; ed. Greene CE, 4th ed., pp. 227. Elsevier Saunders, St. Louis, MO.

Hsieh YC, Lee CC, Tsang CL, Chung YT. Detection and characterization of four novel genotypes of *Ehrlichia canis* from dogs. Vet Microbiol 2010;146:70-75.

Jirapattharasate C, Chatsiriwech J, Suksai P, et al. Identification of *Ehrlichia* spp. in canines in Thailand. Southeast Asian J Trop Med Public Health 2012;43:964-968.

Levy, P.Y., Fenollar, F., The role of molecular diagnostics in implant-associated bone and joint infection. Clin Microbiol Infect 2012;18, 1168-1175.

Maggi, R.G., Compton, S.M., Trull, C.L., Mascarelli, P.E., Mozayeni, B.R., Breitschwerdt, E.B., Infection with hemotropic Mycoplasma species in patients with or without extensive arthropod or animal contact. Journal of clinical microbiology 2013a;51, 3237-3241.

Maggi, R.G., Mascarelli, P.E., Havenga, L.N., Naidoo, V., Breitschwerdt, E.B., Co-infection with Anaplasma platys, Bartonella henselae and Candidatus Mycoplasma haematoparvum in a veterinarian. Parasites & vectors 2013b;6, 103.

Mao, D.P., Zhou, Q., Chen, C.Y., Quan, Z.X., Coverage evaluation of universal bacterial primers using the metagenomic datasets. BMC microbiology 2012;12, 66.

Marin, M., Munoz, P., Sanchez, M., del Rosal, M., Alcala, L., Rodriguez-Creixems, M., Bouza, E. Molecular diagnosis of infective endocarditis by real-time broad-range polymerase chain reaction (PCR) and sequencing directly from heart valve tissue. Medicine (Baltimore) 2007;86, 195-202.

Meinkoth JH, Hoover JP, Cowell RL, et al. Ehrlichiosis in a dog with seizures and nonregenerative anemia. J Am Vet Med Assoc 1989;195:1754-1755.

Mylonakis ME, Kritsepi-Konstantinou M, Dumler JS, et al. Severe hepatitis associated with acute *Ehrlichia canis* infection in a dog. J Vet Intern Med 2010;24:633-638.

Pinyoowong D, Jittapalapong S, Suksawat F, et al. Molecular characterization of Thai *Ehrlichia canis* and *Anaplasma platys* strains detected in dogs. Infect Genet Evol 2008;8:433-438.

Rampini, S.K., Bloemberg, G.V., Keller, P.M., Buchler, A.C., Dollenmaier, G., Speck, R.F., Bottger, E.C. Broad-range 16S rRNA gene polymerase chain reaction for diagnosis of culture-negative bacterial infections. Clin Infect Dis 2011;53, 1245-1251.

Raoult D, Aboudharam G, Crubezy E, et al. Molecular identification by "suicide PCR" of *Yersinia pestis* as the agent of medieval black death. Proc Natl Acad Sci U S A 2000;97:12800-12803.

Schwartz, Z., Zitzer, N.C., Racette, M.A., Manley, P.A., Schaefer, S.L., Markel, M.D., Hao, Z., Holzman, G., Muir, P. Are bacterial load and synovitis related in dogs with inflammatory stifle arthritis? Vet Microbiol 2011;148, 308-316.

Voldstedlund, M., Norum Pedersen, L., Baandrup, U., Klaaborg, K.E., Fuursted, K. Broad-range PCR and sequencing in routine diagnosis of infective endocarditis. APMIS 2008;116, 190-198.

Wang, Y., Qian, P.Y. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. PLoS One 2009;4, e7401.

Woody BJ, Hoskins JD. Ehrlichial diseases of dogs. Vet Clin North Am 1991;21:75-98.

Zweygarth E, Cabezas-Cruz A., Josemans A I, et al. In vitro culture and structural differences in the major immunoreactive protein gp36 of geographically distant *Ehrlichia canis* isolates. Ticks Tick Borne Dis 2014;5:423-431.

7. Appendix

8. Output (Acknowledge the Thailand Research Fund)

8.1 International Journal Publication

Gunn Kaewmongkol, Phudit Maneesaay, Nirut Suwanna, Bordin Tiraphut, Tanhatai Krajarngjang, Anyamanee Chouybumrung, Sarawan Kaewmongkol, Theerapol Sirinarumitr, Sathaporn Jittapalapong and Stanley G. Fenwick. First detection of *Ehrlichia canis* in cerebrospinal fluid from a non-thrombocytopenic dog with meningoencephalitis using broad range PCR. Journal of Veterinary Internal Medicine (Submitted on 1 April 2015, under review)

8.2 Application

8.3 Others e.g. national journal publication, proceeding, international conference, book chapter, patent

Gunn Kaewmongkol, Sarawan Kaewmongkol, Theerapol Sirinarumitr, Sathaporn Jitapalapong' Stanley G. Fenwick. Development of Broad Range Nested-PCR Targeting the 16S rRNA Gene for Detection of Culture-negative Bacteria. The Proceedings of 52nd Kasetsart University Annual Conference 2014: 184-192. (Oral presentation)

Gunn Kaewmongkol, Sarawan Kaewmongkol, Taksaon Duangurai, Kaset Sutasha, Ladawan Areevijitrakul, Theerapol Sirinarumitr, Sathaporn Jittapalapong and Stanley G. Fenwick. Identification of uncultured-bacteria detected from abscesses of pet animals by broad range nested 16S rRNA PCR. The 12th International Conference on Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases; MEEGID XII 2014 (Oral presentation) (Organised by Elsevier Publisher and Infection, Genetic and Evolution is a conference supporting journal held in Bangkok 11-13 December 2014)

Development of Broad Range Nested-PCR Targeting the 16S rRNA Gene for Detection of Culture-negative Bacteria

Gunn Kaewmongkol[1] Sarawan Kaewmongkol[2] Theerapol Sirinarumitr[1] Sathaporn Jitapalapong[1] Stanley G. Fenwick[3]

ABSTRACT

The aim of this study was to develop improved broad-range nested PCR methods to detect bacterial DNA in culture-negative specimens in pet animals presented at Veterinary teaching hospital, Kasetsart University, Bangkok, Thailand. The impetus of this work was initiated because the regularly failures of conventional bacterial isolations have occurred in veterinary practices. In addition, these situations are further complicated by the lack of suitable tools for the detection of culture-negative-bacteria in various clinical specimens. Furthermore, the optimizations of this broad range nested-PCR were performed and the sensitivity of this tool was analyzed in order to clarify the minimal limit for the detection of bacterial DNA. The analytical sensitivity of the nested broad-range PCR targeting the 16S rRNA gene was determined by testing of ten serial 10-fold dilutions which ranged from 10^{6}-10^{-3} bacterial colony forming unit (CFU/ml) and concentration of DNA extracted from each dilution was measured by the Nanodrop^{R} spectrophotometer. The standard serial dilutions were prepared from 6 known bacterial genus and species including *Escherichia coli*, *Staphylococcus* species, *Streptococcus* species, *Pseudomonas* species, *Klebsiella* species and *Proteus* species. According to the analytical sensitivity, the lowest dilution of bacterial culture detected by this tool was 10^{-3} CFU/ml in all bacteria prepared for this study. All PCR products produced by this tool were confirmed by DNA sequencing and these sequencing results demonstrated the correlations between genotypic status of all known bacteria and phenotypic status which included bacterial morphology and biochemical test. DNA extracted from each dilution was measured and DNA concentrations of the lowest dilution (10^{-3} CFU/ml) were ranged from 0.5-1.9 ng/µl.

Key words: Broad Range Nested-PCR16S rRNA Gene Culture-Negative Bacteria

email address: gunn_kaew@yahoo.com, fvetgunn@ku.ac.th

[1]Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand

[2]Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Sean Campus, Nakhon Pathom, Thailand, Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok, Thailand, Center for Advanced Studies for Agricultural and Food, KU Institute for Advanced Studies, Kasetsart University, Bangkok, Thailand (CASAF, NRU-KU, Thailand), Faculty of Veterinary Technology, Kasetsart University, Bangkok, Thailand

[3]School of Veterinary and Biomedical Sciences, Murdoch University, South Street, Murdoch 6150, Western Australia, Australia

INTRODUCTION

Diversity of microorganisms within complex environments has been explored using broad range PCR detections. In the past decade, large numbers of novel bacteria species have been reported using these techniques and these novel species were mainly identified from the ecosystem including soil, water, and plant (Drancourt et al., 2000). These broad range PCRs targeting the 16S rRNA gene have provided the alternative diagnosis in clinical microbiology when conventional bacteriological techniques failed to identify organisms known as culture-negative bacteria (Rampini et al., 2011). A polymicrobial population of bacteria commonly isolated from the environment was detected in a part of inflamed specimens in humans (Marin et al., 2007, Voldstedlund et al., 2008, Schwartz et al., 2011). Consequently, huge numbers of novel pathogens have also been amplified using these broad range PCRs in human specimens that revealed the new era of bacterial infections in human medicine (Schwartz et al., 2011).

Culture-negative specimens used to be defined as sterile inflammation or any inhibitory effects due to antibiotic treatments prior to sample collections (Rampini et al., 2011). Eventually, the detection of bacterial DNA using broad range PCRs has changed previous perceptions of bacterial infections as causes of inflammatory diseases. In contrast to humans, there is less research focusing on the culture-negative bacterial infections in animals, especially in Thailand. To date, very few applications of 16S rRNA PCR have been developed and verified in veterinary medicine. We strongly believe that these unidentified bacterial infections are potentially significant diseases in Thailand that currently go unrecognized in pet animals and these stealth bacteria could also be a potential risk for humans. A total market value for pet business in Thailand was more than 20 billion baht in 2011 (Tharnsettakij, 30 March 2012).

The pet business in Thailand could be categorized into three major parts including pet food products which shared 7 billion baht, pet accessories such as snacks, grooming products and cloths shared 3 billion baht, and veterinary hospital shared 10 billion baht. These results associated with the increased number of new pets and pet owners in Thailand every year (Tharnsettakij, 30 March 2012). The close contact between humans and their pets has been considered as the high risk for disease transmission that could be the source of emerging diseases in human medicine.

The goal of this study was to develop a new broad range nested-PCR for the detection of culture-negative bacteria and this new tool was defined the properties including sensitivity, specificity and repeatability.

MATERIAL AND METHODS

1. Analytical sensitivity and specificity

The analytical sensitivity of the nested broad-range PCR targeting the 16S rRNA gene was determined by testing of ten serial 10-fold dilutions which range from 10^{6}-10^{-3} bacterial colony forming unit (CFU/ml). The standard serial dilutions were prepared using stock culture of known bacterial culture that the known bacterial species performed for this process are listed in Table 1.

Table 1 Organisms used asses the sensitivity and specificity of PCR assays

Organism	Supplier
Escherichia coli	Bacteriology laboratory,
Staphylococcus species	Kasetsart Veterinary Teaching
Streptococcus species	Hospital
Pseudomonas species	
Klebsiella species	
Proteus species	

2. DNA extraction

DNA was extracted from each bacterial dilution (10^{6}-10^{-3} CFU/ml) using QIAamp ® DNA Mini Kits (QIAGEN Inc.) according to the manufacturer's instructions. Following extraction, DNA concentration and purity were measured by Nanodrop^{R} spectrophotometer.

3. Primers, PCR assays and DNA sequencing

PCR primers for the primary PCR were modified based on a previous report (Cai et al., 2013) and consisted of V1-F (5' AGAGTTTGATCCTGGCTCAG 3') and V9-R (5' GNTACCTTGT- TACGACTT 3'). PCR reactions for the primary PCR was performed using 1 µL of DNA in a 25 µL reaction containing 1 x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM of each primer and 0.04 U/µL Taq DNA polymerase (Invitrogen^{R}). The cycling conditions consisted of a pre-PCR step of 95°C for 5 minutes, followed by 40 cycles of 95°C for 60 seconds, 50°C for 60 seconds and an extension of 72°C for 90 seconds, with a final extension of 72°C for 10 minutes. These primary primers produced approximately 1,400 bp PCR products. PCR primers for the secondary PCR consisted of V3-F (5' ACTCCTACGGGAGGCAGCAG 3') and V6-R (5' CGACAGCCATGCANCACCT 3') that these primers were also modified as per a previous study (Lin et al., 2013). The PCR reaction for the secondary PCR were performed using 1 µL of DNA in a 25 µL reaction containing 1 x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM of each primer and 0.04 U/µL Taq DNA polymerase (Invitrogen^{R}). The cycling conditions consisted of a pre-PCR step of 95 °C for 5 minutes, followed by 45 cycles of 95°C for 60 seconds, 55°C for 45 seconds and an extension of 72°C

for 45 seconds with a final extension of 72°C for 10 minutes. These secondary primers produced approximately 700 bp PCR products. All PCR products produced by secondary PCR were purified from agarose gel slices using an UltraClean™ 15 DNA Purification Kit (MO BIO Laboratories Inc. West Carlsbad, California, USA). Sequencing was performed using an ABI Prism™ Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) on an Applied Biosystems 3730 DNA Analyzer, following the manufacturer's instructions.

RESULTS AND DISCUSSION

The nested-PCR technique developed in this study was able to detect bacterial DNA which extracted from bacterial culture with the lowest concentration as 10^{-3} CFU/ml dilution. DNA concentration of all bacterial 10^{-3} CFU/ml dilutions were ranged from 0.5-1.9 ng/µl. Agarose gel electrophoresis results for each bacterial dilution were demonstrated in Fig. 1-6. All 700 bp-PCR products performed for these bacterial species were confirmed by sequencing and the sequencing results were identity to the referenced organisms as described previously.

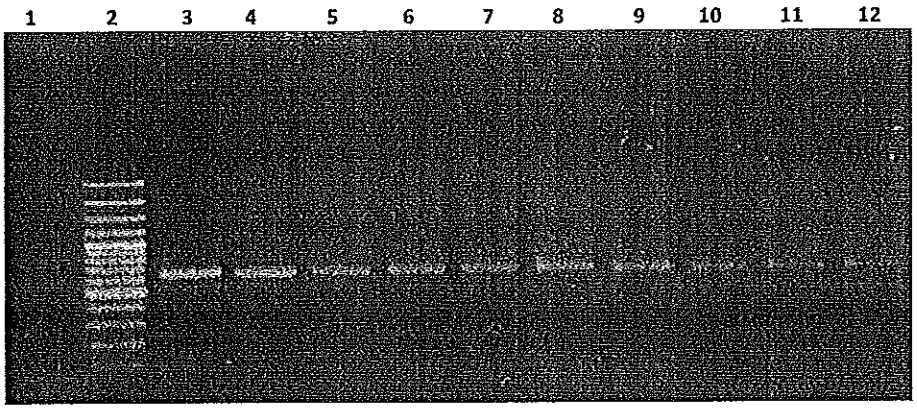

Figure 1 *Proteus* spp. agarose gel electrophoresis. Lane 1: negative control; Lane 2: 100 bp DNA marker; Lane 3-12: *Proteus* spp. in medium culture 10^{6}-10^{-3} CFU/ml, respectively.

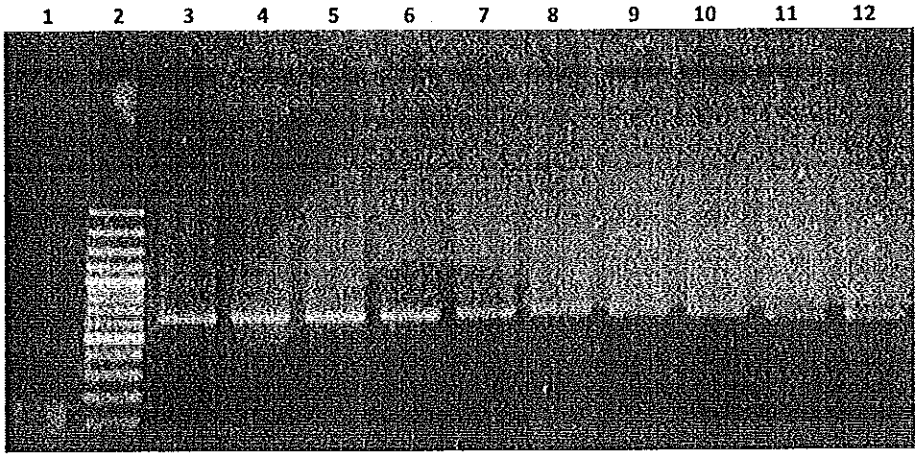

Figure 2 *Streptococcus* spp. agarose gel electrophoresis. Lane 1: negative control; Lane 2: 100 bp DNA marker; Lane 3-12: *Streptococcus* spp in medium culture 10^{6}-10^{-3} CFU/ml, respectively.

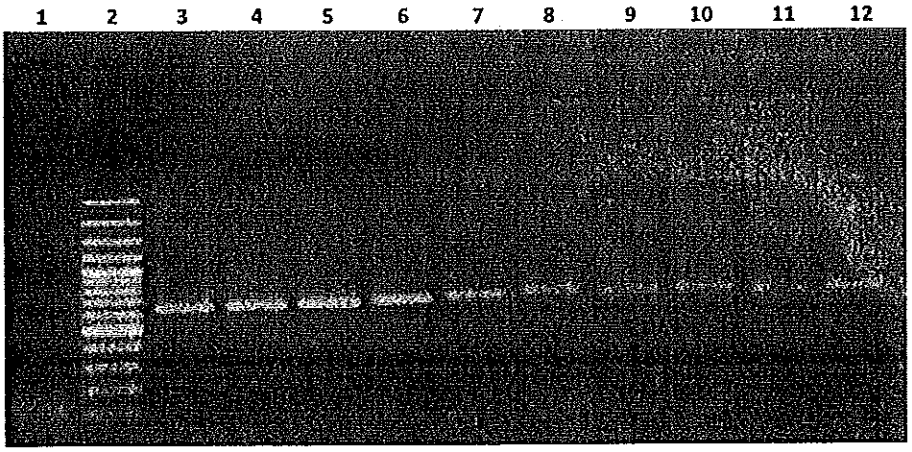

Figure 3 *Staphylococcus* spp. agarose gel electrophoresis. Lane 1: negative control; Lane 2: 100 bp DNA marker; Lane 3-12: *Staphylococcus* spp. in medium culture 10^{6}-10^{-3} CFU/ml, respectively.

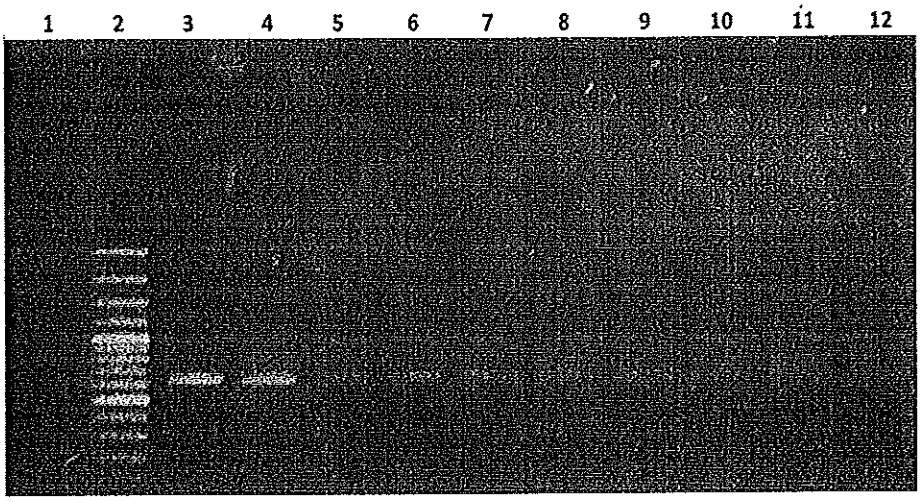

Figure 4 *Klebsiella* spp. agarose gel electrophoresis. Lane 1: negative control; Lane 2: 100 bp DNA marker; Lane 3-12: *Staphylococcus* spp. in medium culture 10^{6}-10^{-3} CFU/ml, respectively.

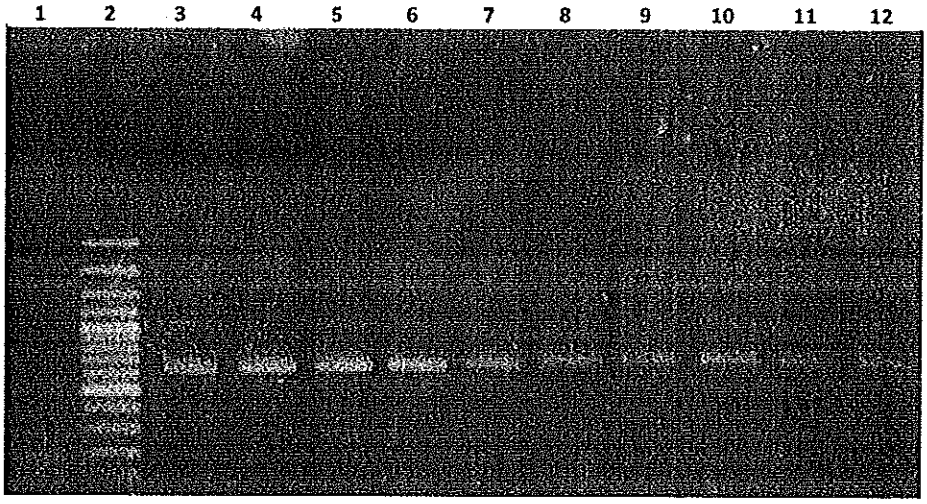

Figure 5 *E. coli* agarose gel electrophoresis. Lane 1: negative control; Lane 2: 100 bp DNA marker; Lane 3-12: *Staphylococcus* spp. in medium culture 10^{6}-10^{-3} CFU/ml, respectively.

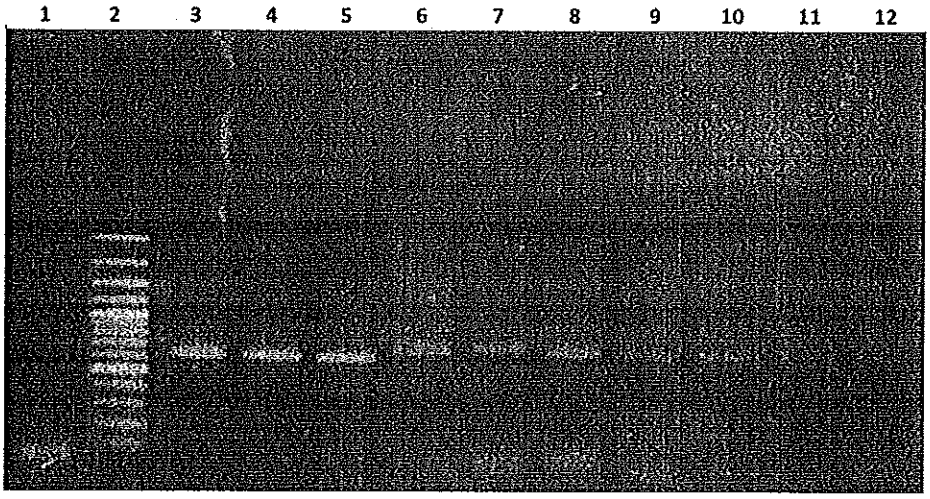

Figure 6 *Pseudomonas* spp. agarose gel electrophoresis. Lane 1: negative control; Lane 2: 100 bp DNA marker; Lane 3-12: *Staphylococcus* spp. in medium culture 10^{6}-10^{-3} CFU/ml, respectively.

The broad range nested-PCR developed in this study presented high sensitivity for the detection of various bacteria species. DNA concentrations were also very low in the lowest bacterial dilutions. However, the correlations between DNA concentration and each bacterial dilution should be performed using statistical analysis in further processes. The concept of the broad-range PCR reported previously has been proved that these tools are actually useful for diagnosis of culture-negative bacterial infections in a variety of clinical specimens (Rampini et al., 2011, Levy et al., 2012, Bosshard et al., 2003). Previous studies of these broad-range PCRs also revealed the huge advantage compared with the conventional bacterial culture techniques. Currently, these tools have been accepted for the applications in culture-negative bacteria in human medicine. However, the bias detections of theses PCRs has been considered that most universal bacterial primers designed in many reports were not able to cover all species of bacteria (Dan-Ping Mao et al., 2012). The coverage evaluation of these 16S rRNA gene primers was proposed (Dan-Ping Mao et al., 2012) and conservative fragments of this gene in the diversity of bacteria were also reported in order to increase the coverage rate of novel primers' designs (Wang et al., 2009). This study created a novel nested-PCR that the primers were modified from the report of conservative fragments in a previous study (Cai et al., 2013). In addition, this study also developed more sensitivity detection using two sets of primers to be a nested-PCR. However, the high sensitivity detection created by this tool could be disadvantage particularly, in cross contaminations by PCR amplicons.

Although this PCR detection will be a useful tool for the diagnosis of culture-negative bacterial infections, antibiotic sensitivity tests are still needed the conventional bacterial culture techniques which could not be replaced by this PCR detection. The results of this broad-range PCR detection will reveal more novel pathogenic bacteria in a variety of clinical specimens in animals that are very important for the improvement of specific culture techniques which require special culture media (solid, semisolid and solution media) and special culture conditions.

ACKNOWLEDGEMENTS

We would like to thank all staffs from Veterinary Diagnostic Laboratory and Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Kasetsart University, Bangkok for their supports in this study. This study was also supported by a grant provided by the Thailand Research Fund, Office of the Higher Education Commission and Kasetsart University Research and Development Institute.

REFERENCES

Bosshard, P.P., Abels, S., Zbinden, R., Bottger, E.C., Altwegg, M., 2003. Ribosomal DNA sequencing for identification of aerobic gram-positive rods in the clinical laboratory (an 18-month evaluation). J Clin Microbiol 41, 4134-4140.

Cai, L., Ye, L., Tong, A.H., Lok, S., Zhang, T., Biased diversity metrics revealed by bacterial 16S pyrotags derived from different primer sets. PLoS One 8, e53649.

Drancourt, M., Bollet, C., Carlioz, A., Martelin, R., Gayral, J.P., Raoult, D., 2000. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J Clin Microbiol 38, 3623-3630.

Levy, P.Y., Fenollar, F., The role of molecular diagnostics in implant-associated bone and joint infection. Clin Microbiol Infect 18, 1168-1175.

Mao, D.P., Zhou, Q., Chen, C.Y., Quan, Z.X., Coverage evaluation of universal bacterial primers using the metagenomic datasets. BMC microbiology 12, 66.

Marin, M., Munoz, P., Sanchez, M., del Rosal, M., Alcala, L., Rodriguez-Creixems, M., Bouza, E., 2007. Molecular diagnosis of infective endocarditis by real-time broad-range polymerase chain reaction (PCR) and sequencing directly from heart valve tissue. Medicine (Baltimore) 86, 195-202.

Rampini, S.K., Bloemberg, G.V., Keller, P.M., Buchler, A.C., Dollenmaier, G., Speck, R.F., Bottger, E.C., 2011. Broad-range 16S rRNA gene polymerase chain reaction for diagnosis of culture-negative bacterial infections. Clin Infect Dis 53, 1245-1251.

Schwartz, Z., Zitzer, N.C., Racette, M.A., Manley, P.A., Schaefer, S.L., Markel, M.D., Hao, Z., Holzman, G., Muir, P., 2011. Are bacterial load and synovitis related in dogs with inflammatory stifle arthritis? Vet Microbiol 148, 308-316.

Voldstedlund, M., Norum Pedersen, L., Baandrup, U., Klaaborg, K.E., Fuursted, K., 2008. Broad-range PCR and sequencing in routine diagnosis of infective endocarditis. APMIS 116, 190-198.

Wang, Y., Qian, P.Y., 2009. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. PLoS One 4, e7401.

Case Report
J Vet Intern Med 2015

First Detection of *Ehrlichia canis* in Cerebrospinal Fluid From a Nonthrombocytopenic Dog with Meningoencephalitis By Broad-Range PCR

G. Kaewmongkol, P. Maneesaay, N. Suwanna, B. Tiraphut, T. Krajarngjang, A. Chouybumrung, S. Kaewmongkol, T. Sirinarumitr, S. Jittapalapong, and S.G. Fenwick

Key words: Broad-range 16S rRNA PCR; Cerebrospinal fluid; *Ehrlichia canis*; Meningoencephalitis.

A 10-year-old, 4.4 kg, intact female poodle presented with clinical signs of weakness in both hind limbs in May 2013 at the Veterinary Teaching Hospital, Kasetsart University, Bangkok, Thailand. The dog had a mild fever (39.5°C) and demonstrated hyperesthesia in the lumbar area. Radiographic imaging showed normal appearance of the spine and intervertebral disk spaces. A CBC was within reference ranges. An antipyretic drug, tolfenamic acid, was administered once SC (4 mg/kg), combined with an analgesic drug, tramadol, administered PO (3 mg/kg). The mild fever improved within 24 hours, but the dog still had signs of weakness in the hind limbs and hyperesthesia in the lumbar area. Intervertebral disk disease at the lumbo-sacral area initially was diagnosed, and anti-inflammatory doses of a corticosteroid were given for 7 days, after which the clinical signs of weakness in both hind limbs had slightly improved. A month after treatment, in June 2013, there were no signs of hind limb weakness, but recurrence of the same clinical signs occurred for 3 months from June to August 2013, and intermittent 7-day courses of a corticosteroid were prescribed at subsequent monthly re-evaluations. On 10th September 2013, the dog was hospitalized with clinical signs of hyperesthesia along the vertebral column and neurologic deficits, including disoriented mental status, unilateral palpebral reflex deficit, and vestibular and cerebellar ataxia. These findings suggested multifocal brain lesions with progressive loss of neurologic functions. A CBC disclosed leukocytosis (26 000 cells/μL) with left shift and mild anemia (hematocrit, 26.8%). Platelet numbers and other blood biochemistry results were normal. Infectious encephalitis was suspected based on the clinical signs and leukocytosis. A sample of cerebrospinal fluid (CSF) was submitted to the diagnostic laboratory for standard bacterial culture and cytologic examination. The results of CSF culture for bacteria using standard techniques were negative. Subsequently, a broad-range nested PCR targeting the 16S rRNA gene was performed to confirm bacterial meningoencephalitis. The dog was treated with amoxicillin/clavulanic acid (30 mg/kg, PO q12h), dexamethasone (0.05 mg/kg, PO q12h), dimenhydrinate (8 mg/kg, PO q12h), and vitamin B.

Abbreviations:

CSF	cerebrospinal fluid
CNS	central nervous system
CME	canine monocytic ehrlichiosis

DNA was extracted from the CSF sample for the PCR using a DNA extraction Kit[a] according to the manufacturer's instructions. A previous study described conserved sequences of the 16S rRNA gene of bacteria that were used for the next-generation DNA sequencing (pyrosequencing)[1]. In the current study, we modified these regions to develop the PCR primers for our novel nested PCR, targeting the 16S rRNA gene (16S rDNA). The analytical sensitivity of the nested broad-range PCR was determined by testing 10 serial 10-fold dilutions of DNA extracted from *Escherichia coli*, *Staphylococcus* spp., *Streptococcus* spp., *Pseudomonas* spp., *Klebsiella* spp., and *Proteus* spp. The dilutions used ranged from 10^{6}–10^{-3} bacterial colony-forming units (CFU/mL) and the concentration of DNA extracted from each dilution was measured by spectrophotometer.[b] The lowest dilution detected by the PCR was 10^{-3} CFU/mL from all bacteria analyzed in this study. DNA concentrations of the lowest dilution (10^{-3} CFU/mL) ranged from 0.5–1.9 ng/μL.

The 16S rDNA primers for the primary PCR consisted of V1-F (5′ AGAGTTTGATCCTGGCTCAG 3′) and V9-R (5′ GNTACCTTGTTACGACTT 3′). The reaction for the primary PCR was performed using 1 μL of DNA in a 25 μL reaction containing 1× PCR buffer, 2 mM

From the Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand (Kaewmongkol, Maneesaay, Suwanna, Sirinarumitr, Jittapalapong); the Neurological Clinic, Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand (Tiraphut, Krajarngjang, Chouybumrung); the Faculty of Veterinary Technology, Kasetsart University, Bangkok, Thailand (Kaewmongkol); and the Department of Infectious Disease and Global Health, Cummings School of Veterinary Medicine, Tufts University, Boston, MA (Fenwick).

Corresponding author: Gunn Kaewmongkol, Faculty of Veterinary Medicine, Kasetsart University, 50 Ngam Wong Wan Rd., Ladyaow Chatuchak 10900, Bangkok, Thailand; e-mails: gunn_kaew@yahoo.com and fvetgunn@ku.ac.th.

Submitted April 1, 2015; Revised September 1, 2015; Accepted October 13, 2015.

DOI: 10.1111/jvim.13788

$MgCl_2$, 0.2 mM dNTPs, 1 μM of each primer, and 0.04 U/μL Taq DNA polymerase.[c] The cycling conditions consisted of a pre-PCR step of 95°C for 5 minutes, followed by 40 cycles of 95°C for 60 seconds, 50°C for 60 seconds, and an extension of 72°C for 90 seconds, with a final extension of 72°C for 10 minutes. Expected length of the primary PCR product was 1400 bp. PCR primers for the secondary PCR consisted of V3-F (5′ ACTCCTACGGGAGGCAGCAG 3′) and V6-R (5′ CGACAGCCATGCANCACCT 3′), also modified from a previous study.[1] The reaction for the secondary PCR was performed using 1 μL of DNA in a 25 μL reaction containing 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of each primer, and 0.04 U/μL Taq DNA polymerase[c]. The cycling conditions consisted of a pre-PCR step of 95°C for 5 minutes, followed by 45 cycles of 95°C for 60 seconds, 55°C for 45 seconds and an extension of 72°C for 45 seconds with a final extension of 72°C for 10 minutes. Expected length of the secondary PCR product was approximately 700 bp. All PCR products were purified from agarose gel slices using a DNA purification kit.[d] Sequencing was performed using a Terminator Cycle Sequencing kit[e] in an Applied Biosystems 3730 DNA Analyzer, following the manufacturer's instructions. The 16S rDNA sequence was analyzed by the Basic Local Alignment Search Tool (BLAST). Bacterial 16S rDNA was detected in the CSF by PCR, and the DNA sequence derived from the PCR product was most closely related to *Ehrlichia canis* in the GenBank database, accession number KC479024, with 99% identity.

Cerebrospinal fluid analysis disclosed an increased protein concentration (197 mg/dL; reference range, 0–30 mg/dL) and mixed cell pleocytosis (34/μL; reference range, 0–5/μL). Cytologic findings included nondegenerative neutrophils, small lymphocytes, mononuclear cells, and macrophages with engulfed red cells, fat droplets, or both. In addition, intracytoplasmic inclusion bodies, consistent with *Ehrlichia* spp. morulae, were found in mononuclear cells. These findings indicated possible meningoencephalitis caused by *E. canis* (Fig 1).

Following the infrequent findings from the BLAST algorithm and CSF analysis, a suicide PCR protocol was adopted to confirm the organism's genetic identity and to prevent cross-contamination of 16S rDNA amplicons.[2] The suicide PCR protocol to overcome the issue of contamination in PCR reactions was first described in a study of the causative agent of the Black Death that killed millions in Western Europe during the 14th century.[2] False-positive PCR results commonly occur in ancient human remnants which have been exposed to and colonized by modern saprophytic microflora. Second and third primer pairs from novel loci were included in the protocol to confirm the identification of *Yersinia pestis* DNA in the ancient human specimens.[2] However, to avoid PCR amplicon cross-contamination, Good Laboratory Practice is most important and should not be replaced by the suicide PCR procedure. The suicide PCR was adopted in this study to confirm the unexpected finding of *E. canis* DNA in the CSF of this unusual clinical case and to rule out potential cross-contamination of the 16S rRNA amplicons. The amplification of a novel gene, the *gp36* of *E. canis*, was performed once without the addition of a positive control. This novel locus had never been amplified previously and was targeted only once with fresh primers. The suicide PCR protocol confirmed that detection by the 16S PCR had not resulted from cross-contamination in our laboratory.

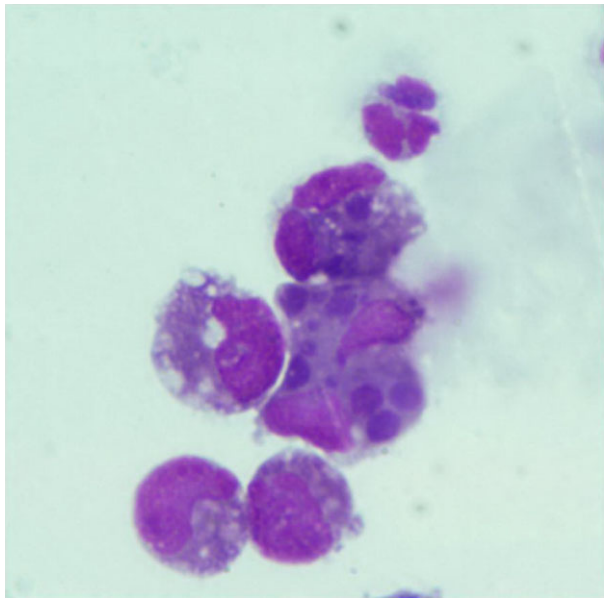

Fig 1. Photomicrograph of cerebrospinal fluid cytology. Mixed cell pleocytosis composed of mononuclear cells, lymphocytes, and neutrophil. Several round-shaped basophilic intracytoplasmic inclusions were seen in mononuclear cells (Wright-Giemsa stain).

The PCR targeting the *gp36* locus of *E. canis* was performed and DNA sequencing of the resulting PCR product was completed after the first amplification. PCR primers for the *gp36* gene, EC36-F1 (5′-GTATGTTTCTTTTATATCATGGC-3′) and EC36-R1 (5′-GGTTATATTTCAGTTATCAGAAG-3′) were used based on a previous study.[3] Nucleotide sequences generated for both loci were analyzed by Chromas lite version 4.0 (http://www.technelysium.com.au) and aligned with reference sequences from *E. canis* from GenBank using Clustal W (http://www.clustalw.genome.jp). A phylogenetic tree of the *gp36* gene was constructed by the distance method and the program Mega version 5.1.[f]

The 16S rRNA and *gp36* loci sequences were submitted to GenBank with accession numbers KM879929 and KM879930, respectively. A neighbor-joining phylogenetic tree of the *gp36* gene was constructed and indicated that the *E. canis*-like genotype detected in the present study was closely related to other validated genotypes reported from Israel, Brazil, United States, Spain, and Nigeria (89.5–96.1% identity; Fig 2). These genotypes were classified as *E. canis* cluster A in a previous report.[4] However, the dog died before advanced brain imaging was performed. The molecular diagnosis and CSF cytology results were recorded subsequently.

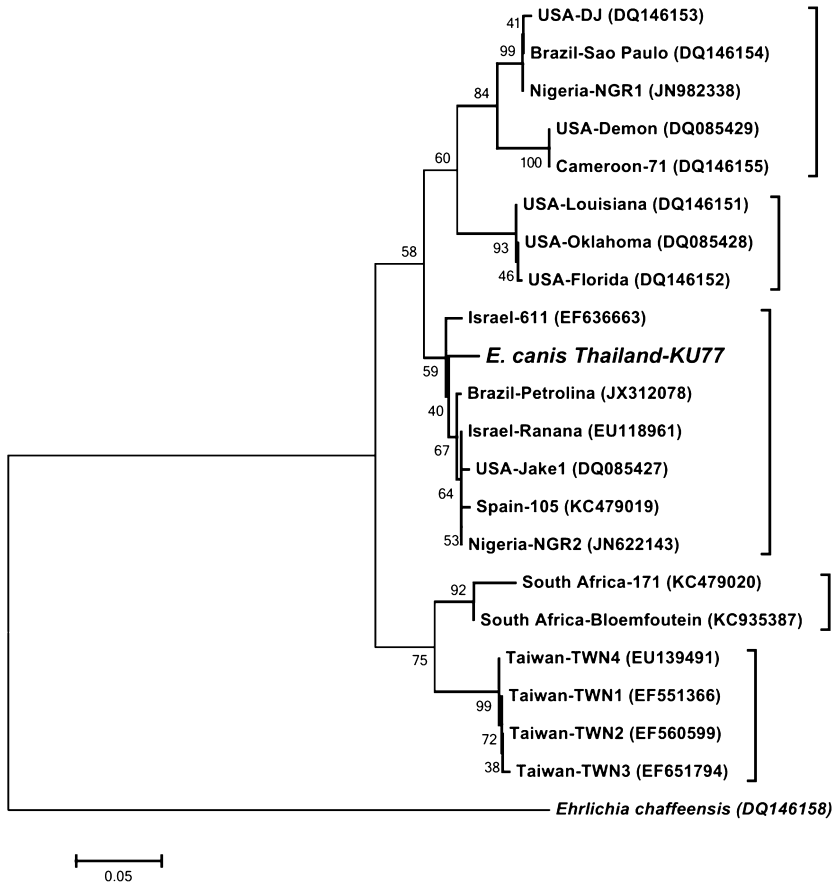

Fig 2. Neighbor-joining phylogenetic tree of the *gp36* gene of *Ehrlichia canis* detected in the cerebrospinal fluid of a dog in Thailand and validated genotypes of *E. canis*. Percentage bootstrap support (>40%) from 1000 pseudoreplicates is indicated at the left of the supported node.

Ehrlichia canis, a tick-borne pathogen, mainly transmitted by *Rhipicephalus sanguineus,* is the cause of canine monocytic ehrlichiosis (CME), which is a multisystemic disease resulting in hematologic abnormalities and respiratory, ocular or neurologic sequelae.[5] Coagulation disorder, because of severe thrombocytopenia and platelet dysfunction, is the most prominent sign of infection with *E. canis*.[5,6] The organism has resulted in high morbidity and mortality in dogs in Southeast Asia since it was first described affecting US military dogs during the Vietnam War in the 1960s.[7] However, published reports in this region, of both clinical manifestations and genetic studies, are very limited.[8–10] Genus-specific primers for Thai *Ehrlichia* and *Anaplasma* were designed based on the 16S rRNA sequences[10]. These genus-specific primers and sequence analysis allow detections of diverse genotypes of the organisms in this region[10] and this report demonstrated advantage of next-generation diagnostics which include broad-range PCR primers and sequence analysis.

Persistent infection of *Ehrlichia muris* in a mouse model was shown to induce antibodies that protected the mice against an ordinarily lethal secondary *Ixodes ovatus Ehrlichia* challenge.[11] In the study, antigen-specific gamma interferon (INF)-producing splenic memory T cells played a major role in the immune protection of the infected mice without doxycycline treatment.[11] Because corticosteroids have been shown to inhibit production of IL-12, a cytokine known to enhance gamma IFN synthesis in mouse splenic adherent cells,[12] the use of corticosteroids in our treatment protocol could have compromised the immune status of this dog, which was most likely in the chronic or subclinical phase of CME, accompanied by persistent infection with *E. canis*. Where possible, diagnosis of chronic or persistent infections of *E. canis* in dogs in endemic areas should be performed before corticosteroid treatment to avoid possible recrudescence of infection. Serodiagnostic tests before corticosteroid treatment of patients have been recommended in strongyloidiasis in humans in endemic regions,[13] because latent infections with the parasite, *Strongyloides stercoralis*, have been described and massive invasion by filariform larvae has been triggered by corticosteroid treatment.[14] In addition, severe strongyloidiasis has been prevented in corticosteroid-treated patients by administration of prophylactic ivermectin.[13] These strategies could be applied for treatment of potential *E. canis* cases in endemic regions and in the future, serodiagnostic tests for *E. canis* will be

recommended before corticosteroid treatment and, if long-term corticosteroids are warranted, prophylactic doxycycline will be used. Chemoprevention programs using doxycycline were performed and validated in French military dogs working in *E. canis* endemic areas.[15] Each dog was administered doxycycline at (3 mg/kg bodyweight, PO q24h) for at least 4 months. The CME mortality and morbidity rates for these 614 dogs in this study were not detected and the seroconversion rate was very low (4%; 24/614). In addition, there were no clinical signs in the seropositive dogs (low titers), and seronegative in these dogs occurred after doxycycline treatment.[15]

This dog showed typical clinical signs of bacterial meningoencephalitis as described. Therefore, initial treatment was based on a diagnosis of bacterial encephalitis. Unfortunately, as a result of the unusual clinical and hematologic findings of this case, *E. canis* was not included in the differential diagnosis and thus initially there were no specific tests used for *E. canis* infection (eg, specific PCR or antibody detection). In retrospect, the rapidly progressing CNS signs in this dog most likely resulted from *E. canis* infection, confirmed by CSF cytology, sequencing result of the 16S rRNA gene, and phylogenetic analysis of the *gp36* gene. Neurologic deficits of ehrlichial meningoencephalitis are influenced by plasma cell infiltration of the meninges or hemorrhage in cerebral or spinal cord parenchyma.[16,17] The first detection of *E. canis* in CSF was reported from the United States in 1989 and the report described seizures as the dominant sign in the infected dog, together with nonregenerative anemia and chronic thrombocytopenia.[18] A subsequent report in 2012 from Japan also described ataxia of the hind limbs in the infected dog, with nonregenerative anemia and severe thrombocytopenia.[16] In addition, the case from Japan reported xanthochromia in the CSF, which is consistent with subarachnoid hemorrhage. There is less information in the literature describing meningoencephalitis associated with ehrlichiosis in dogs without thrombocytopenia and bleeding tendency, as was the case in our patient. A normal platelet count and transient thrombocytopenia were previously reported in a dog with an uncommon case of severe hepatitis associated with acute *E. canis* infection.[19] The use of ampicillin and enrofloxacin to treat this dog was based on an initial diagnosis of infectious hepatitis caused by leptospirosis. *Ehrlichia* spp. morulae were subsequently found after liver impression cytology, and treatment was changed to doxycycline at 7 days postadmission.[19]

The CSF abnormalities reported in our case are nonspecific and easily could be attributed to general bacterial meningoencephalitis. Presumptive diagnosis and treatment in such cases frequently is performed for bacterial or other causes of CSF pleocytosis, and canine erhlichiosis is regularly excluded from the differential diagnosis, particularly in nonthrombocytopenic cases. Consequently, in this case, doxycycline (the drug of choice for CME) was not administered. Although various pathogens could have been eliminated using specific PCR assays for each organism, the broad-range PCR technique we adapted was quicker and more advantageous and should be recommended for the diagnosis of patients with negative CSF culture results, including the diagnosis of atypical CME.

Footnotes

[a] E.Z.N.A.[R] Tissue DNA Kit, Omega Bio-Tek, Inc., Norcross, GA

[b] NanoDrop 1000 Spectrophotometer V3.7, Thermo Fisher Scientific Inc., Wilmington, DE

[c] *Taq* DNA polymerase, Invitrogen[R], Life technologies, Carlsbad, CA

[d] UltraClean[TM] 15 DNA Purification Kit, MO BIO Laboratories Inc., Carlsbad, CA

[e] ABI Prism[TM] Terminator Cycle Sequencing kit, Applied Biosystems, Foster City, CA

[f] Mega5: Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, AZ

Acknowledgments

We thank all staff from the Veterinary Diagnostic Laboratory and Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Kasetsart University, Bangkok for their support in this study. We also thank for Dr Nipon Wongprasert for technical support.

Conflict of Interest Declaration: The authors declare no potential conflicts of interest with respect to the research, authorship, or publication of this article.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

Funding: This study was supported by a grant provided by the Thailand Research Fund, Office of the Higher Education Commission and Kasetsart University Research and Development Institute.

References

1. Cai L, Ye L, Tong AH, et al. Biased diversity metrics revealed by bacterial 16S pyrotags derived from different primer sets. PLoS One 2013;8:e53649.

2. Raoult D, Aboudharam G, Crubezy E, et al. Molecular identification by "suicide PCR" of *Yersinia pestis* as the agent of medieval black death. Proc Natl Acad Sci USA 2000;97:12800–12803.

3. Hsieh YC, Lee CC, Tsang CL, Chung YT. Detection and characterization of four novel genotypes of *Ehrlichia canis* from dogs. Vet Microbiol 2010;146:70–75.

4. Zweygarth E, Cabezas-Cruz A, Josemans AI, et al. In vitro culture and structural differences in the major immunoreactive protein gp36 of geographically distant *Ehrlichia canis* isolates. Ticks Tick Borne Dis 2014;5:423–431.

5. de Castro MB, Machado RZ, de Aquino LP, et al. Experimental acute canine monocytic ehrlichiosis: Clinicopathological and immunopathological findings. Vet Parasitol 2004;119:73–86.

6. Bulla C, Kiomi Takahira R, Pessoa Araujo J Jr, et al. The relationship between the degree of thrombocytopenia and infection with *Ehrlichia canis* in an endemic area. Vet Res 2004;35:141–146.

7. Greene CE. *Ehrlichia* and *Anaplasma* infections. In: Greene CE, ed. Infectious Diseases of the Dog and Cat, 4th ed. St. Louis, MO: Elsevier Saunders; 2012:227.

8. Foongladda S, Inthawong D, Kositanont U, Gaywee J. *Rickettsia*, *Ehrlichia*, *Anaplasma*, and *Bartonella* in ticks and fleas from dogs and cats in Bangkok. Vector Borne Zoonotic Dis 2011;11:1335–1341.

9. Jirapattharasate C, Chatsiriwech J, Suksai P, et al. Identification of *Ehrlichia* spp. in canines in Thailand. Southeast Asian J Trop Med Public Health 2012;43:964–968.

10. Pinyoowong D, Jittapalapong S, Suksawat F, et al. Molecular characterization of Thai *Ehrlichia canis* and *Anaplasma platys* strains detected in dogs. Infect Genet Evol 2008;8:433–438.

11. Thirumalapura NR, Crossley EC, Walker DH, et al. Persistent infection contributes to heterologous protective immunity against fatal ehrlichiosis. Infect Immun 2009;77:5682–5689.

12. De Kruyff RH, Fang Y, Umetsu DT. Corticosteroids enhance the capacity of macrophages to induce Th2 cytokine synthesis in CD4 + lymphocytes by inhibiting IL-12 production. J Immunol 1998;160:2231–2237.

13. Fardet L, Genereau T, Cabane J, et al. Severe strongyloidiasis in corticosteroid-treated patients. Clin Microbiol Infect 2006;12:945–947.

14. Siddiqui AA, Berk SL. Diagnosis of *Strongyloides stercoralis* infection. Clin Infect Dis 2001;33:1040–1047.

15. Davoust B, Keundjian A, Rous V, et al. Validation of chemoprevention of canine monocytic ehrlichiosis with doxycycline. Vet Microbiol 2005;107:279–283.

16. Baba K, Itamoto K, Amimoto A, et al. *Ehrlichia canis* infection in two dogs that emigrated from endemic areas. J Vet Med Sci 2012;74:775–778.

17. Woody BJ, Hoskins JD. Ehrlichial diseases of dogs. Vet Clin North Am Small Anim Pract 1991;21:75–98.

18. Meinkoth JH, Hoover JP, Cowell RL, et al. Ehrlichiosis in a dog with seizures and nonregenerative anemia. J Am Vet Med Assoc 1989;195:1754–1755.

19. Mylonakis ME, Kritsepi-Konstantinou M, Dumler JS, et al. Severe hepatitis associated with acute *Ehrlichia canis* infection in a dog. J Vet Intern Med 2010;24:633–638.