



รายงานวิจัยฉบับสมบูรณ์
(สัญญาเลขที่ R2559B066)

โครงการ

การพัฒนาชุดไพรเมอร์เพื่อการตรวจคัดแยกการติดเชื้อวัณโรคในเป็ดด้วยเทคนิค

Multiplex real-time PCR ควบคู่กับ High-resolution melting analysis

(Development of primer set for differential detection of avian tuberculosis in duck using
Multiplex real-time PCR coupling with High-resolution melting analysis)

โดย

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รายงานวิจัยฉบับสมบูรณ์

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Executive summary

การตรวจพบการติดเชื้อในสิ่งส่งตรวจจากสัตว์ โดยเฉพาะสัตว์เศรษฐกิจในพื้นที่ภาคเหนือตอนล่างเป็นปัญหาสำคัญและส่งผลต่อการมีชีวิตของสัตว์ คุณภาพของผลิตภัณฑ์จากสัตว์ ส่งผลกระทบต่อระบบเศรษฐกิจทั้งการปศุสัตว์และคุณภาพชีวิตของเกษตรกรผู้เลี้ยง การสำรวจข้อมูลโรคติดเชื้อต่างๆ เช่น เชื้อแบคทีเรีย เชื้อปรสิต หรือเชื้อไวรัส ไม่ว่าจะ เป็นข้อมูลชนิดของเชื้อ อุบัติการณ์ ระบาดวิทยา หรือข้อมูลพื้นฐานทางปศุสัตว์จึงมีความสำคัญในการศึกษาวิจัยเพื่อแก้ปัญหาดังกล่าว โดยความร่วมมือของผู้วิจัยทั้งทางชีวเคมี และชีววิทยาโมเลกุล ทางจุลชีววิทยาและปรสิตวิทยา และศูนย์วิจัยและพัฒนาการสัตวแพทย์ (ศวพ.) ภาคเหนือตอนล่าง จ.พิษณุโลก นำโดยผู้อำนวยการศูนย์วิจัย สัตวแพทย์หญิง ดร.จันทร์เพ็ญ ชำนาญพุด และคณะ ซึ่งประกอบไปด้วยนายสัตวแพทย์ชำนาญการด้านการวินิจฉัยโรคติดเชื้อ รวมถึงบุคลากรในสังกัด โดยเฉพาะนักวิทยาศาสตร์การแพทย์ในห้องปฏิบัติการอนุชีววิทยา ได้ตระหนักถึงปัญหาที่เกิดขึ้น โจทย์วิจัยที่สำคัญในพื้นที่ และร่วมปรึกษาหารือเพื่อวิเคราะห์หาแนวทางในการแก้ไขปัญหาที่เกิดขึ้น โดยในเบื้องต้นพบว่า ปัญหาของโรคติดเชื้อที่สำคัญ ได้แก่ การติดเชื้อแบคทีเรีย (*Burkholderia pseudomallei* สาเหตุของโรคmelioidosis และ *Microbacterium* spp. โดยเฉพาะ *M. avium* ในเปิด สาเหตุของโรควัณโรคในเปิด ซึ่งเป็นอุบัติการณ์ใหม่ที่น่าสนใจ) การติดเชื้อปรสิต (*Trypanosoma* spp., *Anaplasma* spp. และ *Babesia* spp. โดยเฉพาะในกลุ่มโคนม ซึ่งพบได้บ่อยครั้งที่มีการตรวจหาการติดเชื้อประจำปีของสถานีวิจัยทดสอบพันธุ์สัตว์พิษณุโลก ตากและนครสวรรค์ หรือ *Fasciola* spp. ไนโค กระบือ) และการติดเชื้อไวรัส (FMD virus สาเหตุของโรคปากเท้าเปื่อย โดยเฉพาะไนโค) ซึ่งจำเป็นจะต้องมีการพัฒนาเทคนิคทางชีววิทยาโมเลกุลในการตรวจหาหรือตรวจคัดแยกการติดเชื้อ เนื่องจากปัญหาทางด้านระบาดวิทยาที่ทำให้การแพร่กระจายของเชื้อที่รวดเร็วและมีโฮสต์ที่สามารถเป็นเจ้าบ้านให้กับเชื้อต่างๆ ได้หลากหลายมากขึ้น นำไปสู่ความรุนแรงของโรค การแพร่กระจายไปในวงกว้างทั้งในกลุ่มสัตว์ด้วยกันเองหรือเกษตรกรผู้เลี้ยง อีกทั้ง มาตรฐานหรือการตรวจยืนยันที่ใช้ในห้องปฏิบัติการ เช่น การย้อมสีดูได้กล้องจุลทรรศน์ การเพาะเลี้ยงเชื้อ หรือแม้กระทั่งเทคนิคทางภูมิคุ้มกันวิทยาเองก็ตาม ก็ยังมีให้ค่าผลบวกและ/หรือลบปลอมได้ ทำให้ค่าความไวและความจำเพาะของการตรวจคัดแยกเชื้อต่ำ ใช้เวลานาน และจำเป็นต้องใช้บุคลากรที่มีความเชี่ยวชาญเป็นอย่างสูงในการตรวจวินิจฉัย และรายงานผล ในการศึกษาวิจัยครั้งนี้ จึงมีจุดประสงค์ที่จะพัฒนาและประยุกต์ใช้เทคนิคทางชีววิทยาโมเลกุลในการตรวจคัดแยกความจำเพาะของการติดเชื้อที่สำคัญ เพื่อเพิ่มความไวและความจำเพาะ ลดระยะเวลาและต้นทุน โดยเฉพาะอย่างยิ่งในกรณีที่เป็นการลงพื้นที่สำรวจและคัดกรองการติดเชื้อในกลุ่มตัวอย่างปริมาณมาก เนื่องจากทาง ศวพ. เอง นอกเหนือจากสิ่งส่งตรวจจากฟาร์ม จากเกษตรกร หรือ จากเจ้าของสัตว์แล้ว ก็ยังมีพันธกิจในการตรวจคัดกรองการติดเชื้อที่สำคัญในสถานีวิจัยทดสอบพันธุ์สัตว์ ในพื้นที่ภาคเหนือตอนล่างอีกด้วย ดังนั้น ทั้งผู้วิจัยและ ศวพ. เองจึงมีความคาดหวังว่าผลงานวิจัยนี้จะเกิดประโยชน์และสามารถนำไปพัฒนาและประยุกต์ใช้ได้จริงในอนาคต

Abstract

Two main animal pathogenic subspecies of *Mycobacterium avium* are *M. avium avium* (Maa) and *M. avium paratuberculosis* (Map). Their pathogenicity is host-specific, Maa causing avian tuberculosis in poultry whereas Map commonly cross-infects to ruminant. Veterinary diagnosis of *M. avium* infections is microscopic examination of acid-fast bacilli or culture in Löwenstein–Jensen medium, which are time-consuming and low sensitivity. This present study aimed to apply real-time PCR coupled with high-resolution melting (HRM) analysis for differential detection of Maa in Thai domestic ducks. Specific primer targeting host-expression dependent (*hed*) region was designed, PCR product of Maa were amplified from duck's tissue lesions whereas Map were amplified from cow and deer. HRM real-time PCR was performed and analyzed. Different HRM patterns were showed and melting temperature were analyzed at $83.26\pm 0.12^{\circ}\text{C}$ for Maa and $84.04\pm 0.09^{\circ}\text{C}$ for Map. This technique can detect as few as 10^2 DNA copies and present high specificity by negative amplification of other pathogenic bacterial species. This technique is sensitive, specific, rapid and does not require fluorescent probes or post-PCR electrophoresis. Our technique is a possible new tool for the detection of Maa and Map infection in tissue specimens.

Keywords: duck; high resolution melting analysis; *Mycobacterium avium*; subspecies

บทนำ

Mycobacterium avium was recognised as a cause of tuberculosis primarily in chickens, ducks and other fowls, but can also infect to an extensive range of different animal species [1]. At least 20 different types of *M. avium* have been identified which cause tuberculosis in most avian species [2]. Avian tuberculosis generally is transmitted by direct contact with infected birds and can susceptible to human and bovine after ingestion of contaminated feed and water. All avian species are susceptible to infection by *M. avium*. Humans, most livestock species, and other mammals can also become infected. The susceptibility is high in domestic fowl (*Gallus domesticus*), pheasants, wild birds, swine, rabbit and human with immunocompromised [3]. In human, there are many authenticated cases of *M. avium* infection although humans are considered highly resistant to this pathogenic species. Infection is more likely to occur in persons with pre-existent diseases, especially those involving the lungs, and in immune systems impaired-person [4].

Infection with *M. avium* causes serious problems in tropical countries in Asia particularly Thailand where a number of infected-poultry including chickens and ducks was reported. There are two main pathogenic subspecies of *M. avium* were recognized as an important problem are *M. avium* subsp. *avium* (Maa) and *M. avium* subsp. *paratuberculosis* (Map).

Several regions in Thailand were presented the overlapping of these two subspecies infections. Avian tuberculosis is one of the most important diseases that affect both domestic and pet birds, the disease is most often caused by *Mycobacterium avium* belonging Maa whereas Map is the etiologic agent of Johne's disease causing chronic diarrhea, malnutrition, and muscular wasting in ruminants [5-6]. Maa and MAP are closely related and similar by morphology or genetics information, and both can infect to the human in the term of *M. avium* complex (MAC). Notably, MAC comprising *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *Silvaticum* or even *M. intracellulare*, may also infect different animal species like cattle, deer, horses, swine, and exotic species besides causing infection in immunocompromised human [7].

Microbiological methods based on microscopic examination of acid-fast bacilli (AFB) presented the low sensitivity regarding to staining procedures and proficiency of technician.

Regularly, the bacterial culture in Löwenstein–Jensen medium is specific to *M. avium* but it's time consuming (6-8 weeks). They are some definite weak points from routine detection protocol, the stained AFB as well as culture colonies of Maa and Map cannot distinguish each other. Furthermore, they have low sensitivity, especially in early or lightly infected cases. High resolution melting (HRM) analysis of DNA is a simple solution for genotyping, scanning of mutation and other aspects on sequence analysis. The melting profile of DNA, a product from PCR amplification, depends on GC content, length, and which is monitored with saturating dyes that fluorescent in the presence of double-stranded DNA [8-9]. In the field of veterinary and animal science, HRM has been used for detection or differentiation of various pathogenic microorganisms for example, in viruses, bacteria, or parasites [10-12]. For mycobacterial, HRM analysis was applied in many aspects, for example, to differential identification at the species level in clinical isolates or in laboratory standard strains from American Type Culture Collection (ATCC) and the Culture Collection [13-14].

For the studies in *mycobacterium avium* such as HRM was applied for identifying the polymorphisms in Map types I, II, and III [15], for sub-typing Map by analysis of short sequence repeats (SSR) loci [16], differentiating Map cattle- and sheep-type [17], and genotyping the members of *M. avium-intracellulare* (MAI) complex [18]. However, real-time Polymerase Chain Reaction (real-time PCR) with HRM for differentiation of Maa and Map in animal lesion specimens has not been reported yet. In the present study, we developed a real-time PCR coupled with HRM to differential detection of Maa and Map in animal lesion specimens including poultry (ducks) and ruminant (cow and deer) hosts. In addition, we also validate the diagnostic specificity and sensitivity of this approach.

Sample collection and research ethic

The animal experiments and related-protocols in this study were approved, the authors were certified by the Institutional Animal Care and Use Committee (Thai-IACUC) cooperated with the Institute of Animal for Scientific Purposes Development (IAD) of Thailand (permit number. U1-05294-2559) and Ethical Principle for the Use and Care of Animals in Scientific Research (ACUC 5/2560), based on the Ethics of Animal Experimentation of the National Research Council of Thailand.

Forty-one DNA samples of *M. avium* infected-domestic ducks were obtained from Veterinary Research and Development Centre (VRDC)-Lower Northern Region, Phitsanulok, Thailand which they were portions of the leftover specimens received from routine laboratory diagnosis. Among 41 DNA samples from ducks, they were isolated from lesions including wings (n=19), lungs (n=9), intestines (n=11) and kidneys (n=2). Two DNA samples of *M. avium* infected cow and deer were kindly provided from National Institute of Animal Health of Thailand which were recorded as isolated from the lesions at lungs of both infected animals. DNA samples of *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*, *Pasteurella multocida* type a, *Salmonella* Group B (derived from microbiology laboratory of VRDC-Lower Northern Region). For negative control, three DNA samples from non-infected ducks and two DNA samples from non-infected cow were used. All DNA samples were stored at -80°C until used.

DNA amplification by Polymerase Chain Reaction (PCR)

For specific DNA amplification, a pair of specific primers were designed to targeting the host-expression dependent (*hed*) region of Maa (GenBank accession number: AJ011837) and Map (GenBank accession number: AJ011838). For the PCR amplification, forward primer was Mav100-F (5'- ATG AAA GCC ATA CCC GAC GTC CCT -3') and reverse primer was Mav100-R (5'- TGC GGT ACT CGA TCA TGC TGT CCT -3'). Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) was used for PCR amplification by the conventional PCR. The total reaction volume was 25 µl with 10x PCR buffer, 50 mM MgCl₂, 10 mM dNTP mix, 10 µM of forward and reverse primers, and Platinum® Taq DNA Polymerase. Using the PCR conditions of 1 cycle for pre-incubation at 95°C for 5 min, 30 cycles of amplification step at 95°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec.

Cloning and sequencing of control DNA

For DNA cloning, sequencing and construction of control plasmids, Positive control plasmids of Maa and Map were constructed by cloning of the relevant PCR products into the pCR[®]2.1-TOPO[®] vector (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions and were subsequently propagated in *Escherichia coli* DH5-alpha strain (Real Biotech Corp., Taipei, Taiwan). After amplification and agarose gel electrophoresis, the bands of PCR product were purified by HiYield[™] Gel/PCR DNA Mini Kit, (Real Biotech Corp., Taipei, Taiwan) and the nucleotide sequence were determined by 3500 Genetic Analyzer (Applied Biosystems, Inc. [Hitachi Ltd], Tokyo, Japan).

High-resolution melting analysis

For Real-time PCR and HRM analysis, LightCycler[®] 96 Real-Time PCR System for detection and analysis were used for amplification and quantification. A LightCycler[®] 480 High Resolution Melting Master (Roche Applied Science, Mannheim, Germany) was used as recommended by the manufacturer, including Master Mix (FastStart Taq DNA Polymerase, reaction buffer, dNTP mix and HRM dye), MgCl₂ and H₂O PCR-grade. The PCR mixture was prepared to contain 1x HRM Master, 2.2 mM MgCl₂, 0.8 μM of each 2 primers (Mav100-F and Mav100-R). The total reaction volume was 20 μl. The PCR cycling for HRM curve acquisition was run under the following conditions: 1 cycle for pre-incubation at 95°C for 10 min, 45 cycles of amplification step at 95°C for 10 sec, 57°C for 8 sec, and 72°C for 15 sec. After amplification, the PCR products were melted by raising the temperature from 60°C to 95°C, with an increment of 0.1°C/sec. After, the amplification curve was monitored real-time, the melting temperatures (*T_m*) and melting curve were determined and analyzed.

Evaluation of sensitivity and specificity of detection

For determination of analytical sensitivity, the ten-folded serial dilution from 10⁹ to 1 copies of Maa and Map positive control plasmids were prepared. For determination of analytical specificity, DNA samples of *P. aeruginosa*, *E. faecalis*, *Escherichia coli*, *Staphylococcus aureus*, *Pasteurella multocida* type a, *Salmonella* Group B were used. They were subsequently analysed by Real-time PCR and HRM analysis as described above. The diagnostic values were calculated using standard methods [19].

Specific amplification of Maa and Map DNA

For PCR amplification and sequencing analysis of *M. avium* DNA. Using the primer targeting the region that described in material and methods, we successfully amplified a predicted 76 bp and 100 bp product specific from DNA of Maa and Map, respectively (Figure 1).

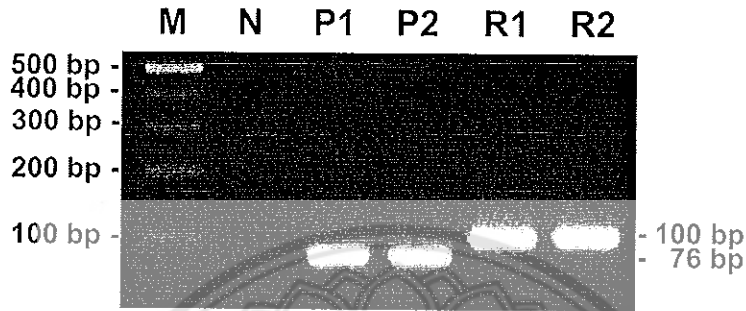


Figure 1: PCR amplification of Maa and Map DNA. The 1.5% agarose gel electrophoresis presents 76 bp and 100 bp PCR products indicating Maa and Map DNA, respectively. For each land, M: 100 bp DNA ladder; N: Negative control (no DNA template); P1 and P2: DNA samples from Maa infected ducks; R1 and R2: DNA samples from Map infected cow and deer, respectively.

After purification and DNA sequencing, the obtained sequences were matched to the sequences of genes from which the primers were designed. There are some deletions occur in the region of Maa DNA that amplified from duck’s infected-tissue compared with 100 bp of Map DNA from infected tissue of the cow and the deer (Figure 2).

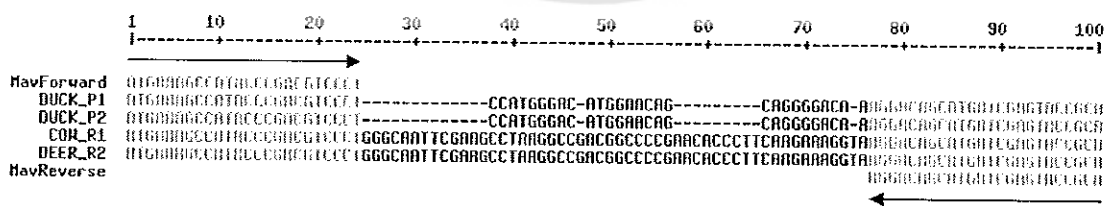


Figure 2: Primer design and alignment with Maa and Map sequences obtained from DNA sequencing. Alignment of Mav100 forward and reverse primers with the sequence obtained from sequencing of amplification products from DNA samples of Maa infected ducks (P1 and

P2), Map infected cow (R1) and Map infected deer (R2). The arrows indicate the direction of each primer for PCR amplification.

For HRM real-time PCR for differential detection of Maa and Map, the method yielded the positive results for all 41 Maa-infected and 2 Map-infected DNA samples. After amplification, the melting temperatures (T_m) and melting curve were analysed. Representative melting temperatures were $83.26 \pm 0.12^\circ\text{C}$ for Maa and $84.04 \pm 0.09^\circ\text{C}$ for Map (Figure 3A-C). DNA from Maa and Map could be obviously distinguished by the specific melting temperatures determined by the difference plots (Figure 3A), the normalized melting curves (Figure 3B), and the melting peaks (Figure 3C).

To standardization of the HRM real-time PCR, there is no fluorescent signal were detected when evaluated with the no DNA control of each experiments, especially in 3 DNA samples from non-infected ducks and 2 DNA samples from non-infected cow. For analytical specificity of HRM real-time PCR, there are no positive result from amplicon at specific melting temperatures was perceived when various DNAs from other pathogenic bacterial species were analysed which demonstrates the specificity of this method (Figure 3D). In addition, the analytical sensitivity was determined using ten-fold serial dilutions (10^9 - 1 copies) of the Maa and Map positive control plasmids. The sensitivity was illustrated by DNA of both Maa and Map could be detected at the lowest concentration used (10^2 copies) when considering 45 cycles as the cut-off detection limit (Figure 3E-F).

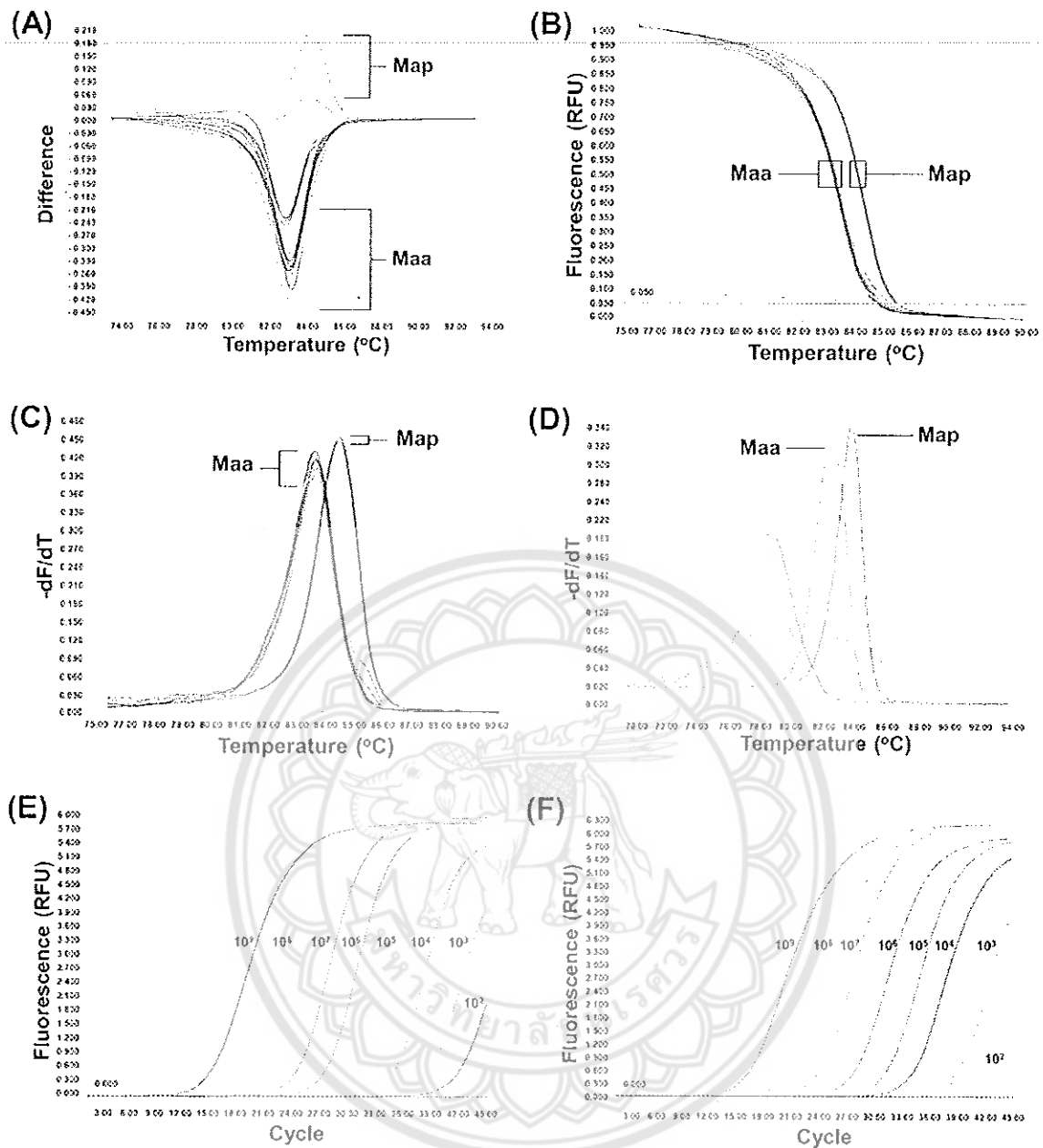


Figure 3: HRM analyses of amplification products for differentiation, and standardization of HRM real-time PCR for detection of Maa and Map. The difference plots (A), normalized melting curves (B) and melting peaks (C) show the different melting temperatures of Maa and Map DNA amplified. Melting peak analyses shows the specificity for differential detection of Maa and Map from the other tested bacterial species as described in the Materials and Methods section (D) and the amplification curves presents the analytical sensitivity for detecting the 10-folded serial dilution plasmids (from 10^9 to 10^2 copies) of Maa (E) and Map (F).



This is the report of HRM real-time PCR to distinguish between Maa and Map in DNA samples extracted from tissue lesion specimens of infected animals in a single assay. We present the application for homogenous genotyping of *M. avium* subspecies without fluorescent labelled-probe and post-PCR electrophoresis. Therefore, the method consequently reducing the expense on a cost per sample and the time consuming. Simplification of interpretation is obtained by different sequences from different subspecies represent a change in the temperature in analysed-melting peak. The HRM real-time PCR offers a new alternative for rapid, sensitive and subspecies specific for differentiation and detection of two main pathogenic *M. avium* subspecies in poultry and ruminant hosts. The high sensitivity and specificity suggest the present test will be of diagnostic value. The analytical sensitivity in this study was only 100 copies of Maa and Map DNA. Moreover, real-time PCR with HRM produced no cross reaction between Maa and Map or various DNAs from other bacterial species that usually infected duck and cow indicating high specificity.

There are several studies mention the application of HRM real-time PCR for *mycobacterial*. Recently in 2017, the researchers defined comparison of melting curves of different non-tuberculosis mycobacteria and presented the melting curves corresponding to the 6 unique-specific HRM groups (*M. tuberculosis* complex, *M. kansasii*, *M. simiae*, *M. fortuitum*, *M. abscessus*-*M. chelonae* group, and *M. avium* complex) for the mycobacterial *rpoBC* locus in several collected clinical specimens [13].

Previously, the standardization of HRM real-time PCR were verified by laboratory standard of *Mycobacterial* strains [14]. In that study the DNA were amplified by primer targeting 16S rRNA gene, the T_m values for *M. tuberculosis* and *M. bovis* of the were $86.00 \pm 0.00^\circ\text{C}$. *M. smegmatis*, *M. ulcerans* and *M. xenopi*, the T_m values were $86.40 \pm 0.00^\circ\text{C}$, $86.20 \pm 0.00^\circ\text{C}$ and $86.60 \pm 0.00^\circ\text{C}$, respectively. The T_m values for *M. avium*, *M. avium* subspecies *paratuberculosis* and *M. intracellulare* of the *M. avium* complex (MAC) were $86.20 \pm 0.00^\circ\text{C}$, $86.60 \pm 0.00^\circ\text{C}$ and $86.80 \pm 0.00^\circ\text{C}$, respectively. In our present study, we use the primer targeting at *hed* region and the T_m values at $83.26 \pm 0.12^\circ\text{C}$ for Maa and $84.04 \pm 0.09^\circ\text{C}$ for Map were obtained. We presented practically almost 1°C for distinguish of Maa and Map whereas previous study is only 0.4°C . In addition, DNA samples of the related pathogenic bacterial species in this group of animals, the test did not give rise to an identifiable T_m or melting peak of Maa and Map, indicating the specificity of this method.

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กิตติกรรมประกาศ

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1. Thorel MF, Huchzermeyer H, Weiss R, Fontaine JJ. *Mycobacterium avium* infections in animals: Literature review. *Vet. Res.* 1997; 28: 439-447.
2. Thorel MF, Krichevsky M, Lévy-Frébault VV. Numerical taxonomy of mycobactin-dependent mycobacteria, amended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. *nov.*, *Mycobacterium avium* subsp. *paratuberculosis* subsp. *nov.*, and *Mycobacterium avium* subsp. *silvaticum* subsp. *nov.*. *Int. J. Syst. Bacteriol.* 1990; 40: 254-260.
3. Dhama K, Mahendran M, Tiwari R, Singh SD, Kumar D, Singh S, Sawant PM. Tuberculosis in birds: Insights into the *Mycobacterium avium* infections. *Vet. Med. Int.* 2011; 2011: 712369.
4. Ramirez J, Mason C; Ali J, Lopez FA. *Mycobacterium avium* complex pulmonary disease: management options in HIV-negative patient. *J. La State Med. Soc.* 2008; 160: 248-254.
5. Mijs W, De Haas P, Rossau R, van Der Laan T, Rigouts L, Portaels F, van Soolingen D. Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* for bird-type isolates and *M. avium* subsp. *hominissuis* for the human/porcine type of *M. avium*. *Int. J. Syst. Evol. Microbiol.* 2002; 52: 1505-1518.
6. Rathnaiah G, Zinniel DK, Bannantine JP, Stabel JR, Gröhn YT, Collins MT, Barletta RG. Pathogenesis, molecular genetics, and genomics of *Mycobacterium avium* subsp. *paratuberculosis*, the etiologic agent of Johne's disease. *Front. Vet. Sci.* 2017; 4: 187.
7. Thorel MF, Huchzermeyer H, Michel AL. *Mycobacterium avium* and *M. intracellulare* infection in mammals. *Rev. Sci. Tech.* 2001; 20: 204-218.
8. Reed GH, Kent JO, Wittwer CT. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics.* 2007; 8: 597-608.
9. Tong SY, Giffard PM. Microbiological applications of high-resolution melting analysis. *J. Clin. Microbiol.* 2012; 50: 3418-3421.
10. Chamings A, Hewson KA, O'Rourke D, Ignjatovic J, Noormohammadi AH. High-resolution melt curve analysis to confirm the presence of co-circulating isolates of avian nephritis virus in commercial chicken flocks. *Avian Pathol.* 2015; 44: 443-451.
11. Miller M, Zorn J, Brielmeier M. High-resolution melting curve analysis for identification of *Pasteurellaceae* species in experimental animal facilities. *PLoS One.* 2015; 10: e0142560.

12. Santos GB, Espínola SM, Ferreira HB, Margis R, Zaha A. Rapid detection of *Echinococcus* species by a high-resolution melting (HRM) approach. *Parasit. Vectors.* 2013; 14: 327.
13. Khosrvai AD, Hashemzadeh M, Hashemi-Shahraki A, Teimoori A. Differential identification of *Mycobacterial* subspecies using high-resolution melting analysis. *Front. Microbiol.* 2017; 23: 2045.
14. Issa R, Abdul H, Hashim SH, Seradja VH, Shaili N, Hassan NA. High resolution melting analysis for the differentiation of *Mycobacterium species*. *J. Med. Microbiol.* 2014; 63: 1284-1287.
15. Castellanos E, Aranaz A, De Buck J. PCR amplification and high-resolution melting curve analysis as a rapid diagnostic method for genotyping members of the *Mycobacterium avium-intracellulare* complex. *Clin. Microbiol. Infect.* 2010; 16: 1658-1662.
16. Ricchi M, Barbieri G, Cammi G, Garbarino CA, Arrigoni N. High-resolution melting for analysis of short sequence repeats in *Mycobacterium avium* subsp. *paratuberculosis*. *FEMS Microbiol. Lett.* 2011; 323: 151-154.
17. Douarre PE, Cashman W, Buckley J, Coffey A, O'Mahony JM. High resolution melting PCR to differentiate *Mycobacterium avium* subsp. *paratuberculosis* cattle type and sheep type. *J. Microbiol. Methods.* 2011; 88: 172-174.
18. Castellanos E, Aranaz A, De Buck J. Rapid identification and differentiation of *Mycobacterium avium* subspecies *paratuberculosis* types by use of real-time PCR and high-resolution melt analysis of the MAP1506 locus. *J. Clin. Microbiol.* 2010; 48: 1474-1477.
19. Galen RS. Predictive value and efficiency of laboratory testing. *Pediatr. Clin. North. Am.* 1980; 27: 861-869.

ภาคผนวก

บทความสำหรับการเผยแพร่

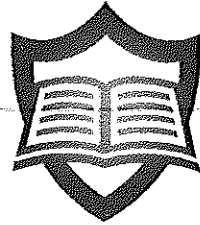
Kaewkong K, Suwanmanee G, Pothipan P, Purisotayo T, Singjam S. Real-time polymerase chain reaction coupled with high-resolution melting analysis for the differential detection of *Mycobacterium avium* subspecies *avium* versus *Mycobacterium avium* subspecies *paratuberculosis* in animal lesion specimens. American J Current App Res Microbiol. 2019; 1(1): 17-021.

วิทยากรรับเชิญ (Invited speaker)

บรรยาย หัวข้อเรื่อง Detection strategies under molecular techniques of *Mycobacterium* infection in poultry host ในการประชุมวิชาการระดับนานาชาติ The 3rd MSU Veterinary International Conference “Animal health and production” วันที่ 17-18 กรกฎาคม 2560 คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหาสารคาม

การนำเสนอผลงาน (โปสเตอร์)

นำเสนอโปสเตอร์ หัวข้อเรื่อง Development of Subspecies Differentiation of *Mycobacterium avium* using High-resolution melting real-time PCR ใน National Genetics Conference 2015; NGC 2015) วันที่ 15-17 กรกฎาคม 2558 ณ โรงแรมเซ็นทารา แอนด์ คอนเวนชันเซ็นเตอร์ ขอนแก่น



American Journal of Current & Applied Research in Microbiology

Research Article

Real-Time Polymerase Chain Reaction Coupled With High-Resolution Melting Analysis for the Differential Detection of *Mycobacterium Avium* Subspecies *Avium* versus *Mycobacterium Avium* Subspecies *Paratuberculosis* in Animal Lesion Specimens - 6

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ABSTRACT

Two main animal pathogenic subspecies of *Mycobacterium avium* are *M. avium avium* (Maa) and *M. avium paratuberculosis* (Map). Their pathogenicity is host-specific, Maa causing avian tuberculosis in poultry whereas Map commonly cross-infects to ruminant. Veterinary diagnosis of *M. avium* infections is microscopic examination of acid-fast bacilli or culture in Löwenstein-Jensen medium, which are time-consuming and low sensitivity. This present study aimed to apply real-time PCR coupled with High-Resolution Melting (HRM) analysis for differential detection of Maa in Thai domestic ducks. Specific primer targeting host-expression dependent (*hcd*) region was designed, PCR product of Maa were amplified from duck's tissue lesions whereas Map were amplified from cow and deer. HRM real-time PCR was performed and analyzed. Different HRM patterns were showed and melting temperature were analyzed at $83.26 \pm 0.12^\circ\text{C}$ for Maa and $84.04 \pm 0.09^\circ\text{C}$ for Map. This technique can detect as few as 10^2 DNA copies and present high specificity by negative amplification of other pathogenic bacterial species. This technique is sensitive, specific, rapid and does not require fluorescent probes or post-PCR electrophoresis. Our technique is a possible new tool for the detection of Maa and Map infection in tissue specimens.

Keywords: Duck; High resolution melting analysis; *Mycobacterium Avium*; Subspecies

INTRODUCTION

Mycobacterium avium was recognised as a cause of tuberculosis primarily in chickens, ducks and other fowls, but can also infect to an extensive range of different animal species [1]. At least 20 different types of *M. avium* have been identified which cause tuberculosis in most avian species [2]. Avian tuberculosis generally is transmitted by direct contact with infected birds and can susceptible to human and bovine after ingestion of contaminated feed and water. All avian species are susceptible to infection by *M. avium*. Humans, most livestock species, and other mammals can also become infected. The susceptibility is high in domestic fowl (*Gallus domesticus*), pheasants, wild birds, swine, rabbit and human with immunocompromised [3]. In human, there are many authenticated cases of *M. avium* infection although humans are considered highly resistant to this pathogenic species. Infection is more likely to occur in persons with pre-existent diseases, especially those involving the lungs, and in immune systems impaired-person [4].

Infection with *M. avium* causes serious problems in tropical countries in Asia particularly Thailand where a number of infected-poultry including chickens and ducks was reported. There are two main pathogenic subspecies of *M. avium* were recognized as an important problem are *M. avium* subsp. *avium* (Maa) and *M. avium* subsp. *paratuberculosis* (Map).

Several regions in Thailand were presented the overlapping of these two subspecies infections. Avian tuberculosis is one of the most important diseases that affect both domestic and pet birds, the disease is most often caused by *Mycobacterium avium* belonging Maa whereas Map is the etiologic agent of Johne's disease causing chronic diarrhea, malnutrition, and muscular wasting in ruminants [5,6]. Maa and MAP are closely related and similar by morphology or genetics information, and both can infect to the human in the term of *M. Avium* Complex (MAC). Notably, MAC comprising *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *Silvaticum* or even *M. intracellulare*, may also infect different animal species like cattle, deer, horses, swine, and exotic species besides causing infection in immunocompromised human [7].

Microbiological methods based on microscopic examination of Acid-Fast Bacilli (AFB) presented the low sensitivity regarding to staining procedures and proficiency of technician. Regularly, the bacterial culture in Lowenstein-Jensen medium is specific to *M. avium* but it's time consuming (6-8 weeks). They are some definite weak points from routine detection protocol, the stained AFB as well as culture colonies of Maa and Map cannot distinguish each other.

Furthermore, they have low sensitivity, especially in early or lightly infected cases.

High Resolution Melting (HRM) analysis of DNA is a simple solution for genotyping, scanning of mutation and other aspects on sequence analysis. The melting profile of DNA, a product from PCR amplification, depends on GC content, length, and which is monitored with saturating dyes that fluorescent in the presence of double-stranded DNA [8,9]. In the field of veterinary and animal science, HRM has been used for detection or differentiation of various pathogenic microorganisms for example, in viruses, bacteria, or parasites [10-12]. For mycobacterial, HRM analysis was applied in many aspects, for example, to differential identification at the species level in clinical isolates or in laboratory standard strains from American Type Culture Collection (ATCC) and the Culture Collection [13,14].

For the studies in *mycobacterium avium* such as HRM was applied for identifying the polymorphisms in Map types I, II, and III [15], for sub-typing Map by analysis of Short Sequence Repeats (SSR) loci [16], differentiating Map cattle- and sheep-type [17], and genotyping the members of *M. Avium-Intracellulare* (MAI) complex [18]. However, real-time Polymerase Chain Reaction (real-time PCR) with HRM for differentiation of Maa and Map in animal lesion specimens has not been reported yet. In the present study, we developed a real-time PCR coupled with HRM to differential detection of Maa and Map in animal lesion specimens including poultry (ducks) and ruminant (cow and deer) hosts. In addition, we also validate the diagnostic specificity and sensitivity of this approach.

MATERIALS AND METHODS

Sample collection and research ethic

The animal experiments and related-protocols in this study were approved, the authors were certified by the Institutional Animal Care and Use Committee (Thai-IACUC) cooperated with the Institute of Animal for Scientific Purposes Development (IAD) of Thailand (permit number. U1-05294-2559) and Ethical Principle for the Use and Care of Animals in Scientific Research (ACUC 5/2560), based on the Ethics of Animal Experimentation of the National Research Council of Thailand.

Forty-one DNA samples of *M. avium* infected-domestic ducks were obtained from Veterinary Research and Development Centre (VRDC)-Lower Northern Region, Phitsanulok, Thailand which they were portions of the leftover specimens received from routine laboratory diagnosis. Among 41 DNA samples from ducks, they were

isolated from lesions including wings (n = 19), lungs (n = 9), intestines (n = 11) and kidneys (n = 2). Two DNA samples of *M. avium* infected cow and deer were kindly provided from National Institute of Animal Health of Thailand which were recorded as isolated from the lesions at lungs of both infected animals. DNA samples of *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*, *Pasteurella multocida* type a, *Salmonella* Group B (derived from microbiology laboratory of VRDC-Lower Northern Region). For negative control, three DNA samples from non-infected ducks and two DNA samples from non-infected cow were used. All DNA samples were stored at -80°C until used. DNA amplification by Polymerase Chain Reaction (PCR)

For specific DNA amplification, a pair of specific primers were designed to targeting the host-expression dependent (*hed*) region of *Maa* (GenBank accession number: AJ011837) and *Map* (GenBank accession number: AJ011838). For the PCR amplification, forward primer was Mav100-F (5'- ATG AAA GCC ATA CCC GAC GTC CCT -3') and reverse primer was Mav100-R (5'- TGC GGT ACT CGA TCA TGC TGT CCT -3'). Platinum[®] Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) was used for PCR amplification by the conventional PCR. The total reaction volume was 25 µl with 10x PCR buffer, 50 mM MgCl₂, 10 mM dNTP mix, 10 µM of forward and reverse primers, and Platinum[®] Taq DNA Polymerase. Using the PCR conditions of 1 cycle for pre-incubation at 95°C for 5 min, 30 cycles of amplification step at 95°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec.

Cloning and sequencing of control DNA

For DNA cloning, sequencing and construction of control plasmids, Positive control plasmids of *Maa* and *Map* were constructed by cloning of the relevant PCR products into the pCR2.1-TOPO[®] vector (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions and were subsequently propagated in *Escherichia coli* DH5-alpha strain (Real Biotech Corp., Taipei, Taiwan). After amplification and agarose gel electrophoresis, the bands of PCR product were purified by HiYield[™] Gel/PCR DNA Mini Kit, (Real Biotech Corp., Taipei, Taiwan) and the nucleotide sequence were determined by 3500 Genetic Analyzer (Applied Biosystems, Inc. [Hitachi Ltd], Tokyo, Japan).

High-resolution melting analysis

For Real-time PCR and HRM analysis, LightCycler[®] 96 Real-Time PCR System for detection and analysis were used for amplification and quantification. A LightCycler[®] 480 High Resolution Melting Master (Roche Applied Science, Mannheim, Germany) was used as recommended by the manufacturer, including Master Mix (FastStart Taq DNA Polymerase, reaction buffer, dNTP mix and HRM dye), MgCl₂ and H₂O PCR-grade. The PCR mixture was prepared to contain 1x HRM Master, 2.2 mM MgCl₂, 0.8 µM of each 2 primers (Mav100-F and Mav100-R). The total reaction volume was 20 µl. The PCR cycling for HRM curve acquisition was run under the following conditions: 1 cycle for pre-incubation at 95°C for 10 min, 45 cycles of amplification step at 95°C for 10 sec, 57°C for 8 sec, and 72°C for 15 sec. After amplification, the PCR products were melted by raising the temperature from 60°C to 95°C, with an increment of 0.1°C/sec. After, the amplification curve was monitored real-time, the melting temperatures (*T_m*) and melting curve were determined and analyzed.

Evaluation of sensitivity and specificity of detection

For determination of analytical sensitivity, the ten-folded serial dilution from 10⁹ to 1 copies of *Maa* and *Map* positive control plasmids

were prepared. For determination of analytical specificity, DNA samples of *P. aeruginosa*, *E. faecalis*, *Escherichia coli*, *Staphylococcus aureus*, *Pasteurella multocida* type a, *Salmonella* Group B were used. They were subsequently analysed by Real-time PCR and HRM analysis as described above. The diagnostic values were calculated using standard methods [19].

RESULTS

Specific amplification of *Maa* and *Map* DNA

For PCR amplification and sequencing analysis of *M. avium* DNA. Using the primer targeting the region that described in material and methods, we successfully amplified a predicted 76 bp and 100 bp product specific from DNA of *Maa* and *Map*, respectively (Figure 1).

After purification and DNA sequencing, the obtained sequences were matched to the sequences of genes from which the primers were designed. There are some deletions occur in the region of *Maa* DNA that amplified from duck's infected-tissue compared with 100 bp of *Map* DNA from infected tissue of the cow and the deer (Figure 2).

For HRM real-time PCR for differential detection of *Maa* and *Map*, the method yielded the positive results for all 41 *Maa*-infected and 2 *Map*-infected DNA samples. After amplification, the melting temperatures (*T_m*) and melting curve were analysed. Representative melting temperatures were 83.26 ± 0.12°C for *Maa* and 84.04 ± 0.09°C for *Map* (Figure 3A-C). DNA from *Maa* and *Map* could be obviously distinguished by the specific melting temperatures determined by the difference plots (Figure 3A), the normalized melting curves (Figure 3B), and the melting peaks (Figure 3C).

To standardization of the HRM real-time PCR, there is no fluorescent signal were detected when evaluated with the no DNA control of each experiments, especially in 3 DNA samples from non-infected ducks and 2 DNA samples from non-infected cow. For analytical specificity of HRM real-time PCR, there are no positive

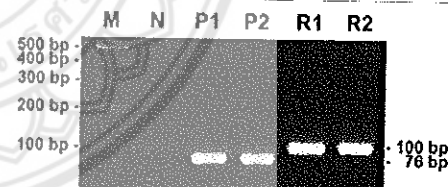


Figure 1: PCR amplification of *Maa* and *Map* DNA. The 1.5% agarose gel electrophoresis presents 76 bp and 100 bp PCR products indicating *Maa* and *Map* DNA, respectively. For each lane, M: 100 bp DNA ladder; N: Negative control (no DNA template); P1 and P2: DNA samples from *Maa* infected ducks; R1 and R2: DNA samples from *Map* infected cow and deer, respectively.

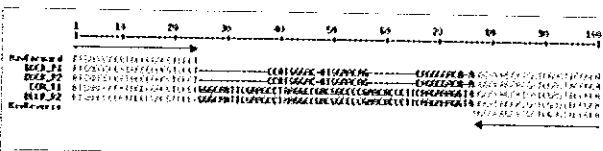


Figure 2: Primer design and alignment with *Maa* and *Map* sequences obtained from DNA sequencing. Alignment of Mav100 forward and reverse primers with the sequence obtained from sequencing of amplification products from DNA samples of *Maa* infected ducks (P1 and P2), *Map* infected cow (R1) and *Map* infected deer (R2). The arrows indicate the direction of each primer for PCR amplification.

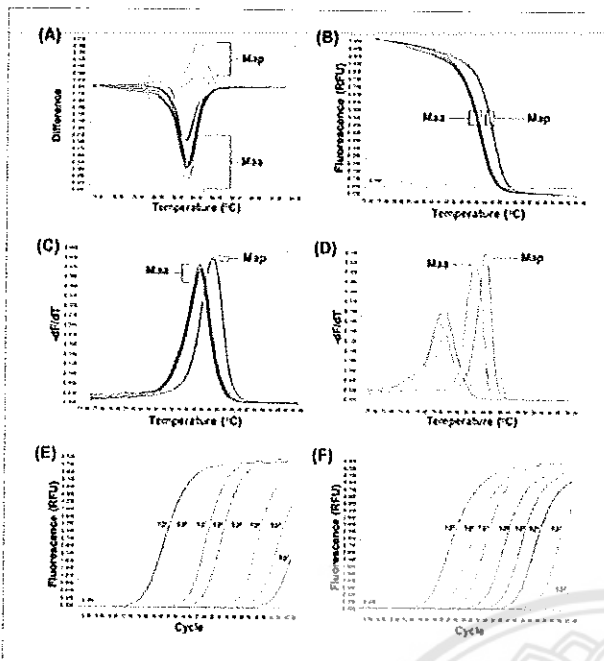


Figure 3: HRM analyses of amplification products for differentiation, and standardization of HRM real-time PCR for detection of Maa and Map. The difference plots (A), normalized melting curves (B) and melting peaks (C) show the different melting temperatures of Maa and Map DNA amplified. Melting peak analyses shows the specificity for differential detection of Maa and Map from the other tested bacterial species as described in the Materials and Methods section (D) and the amplification curves presents the analytical sensitivity for detecting the 10³-fold serial dilution plasmids (from 10⁹ to 10² copies) of Maa (E) and Map (F).

result from amplicon at specific melting temperatures was perceived when various DNAs from other pathogenic bacterial species were analysed which demonstrates the specificity of this method (Figure 3D). In addition, the analytical sensitivity was determined using ten-fold serial dilutions (10⁹ - 1 copies) of the Maa and Map positive control plasmids. The sensitivity was illustrated by DNA of both Maa and Map could be detected at the lowest concentration used (10² copies) when considering 45 cycles as the cut-off detection limit (Figure 3E-F).

DISCUSSION

This is the report of HRM real-time PCR to distinguish between Maa and Map in DNA samples extracted from tissue lesion specimens of infected animals in a single assay. We present the application for homogenous genotyping of *M. avium* subspecies without fluorescent labelled-probe and post-PCR electrophoresis. Therefore, the method consequently reducing the expense on a cost per sample and the time consuming. Simplification of interpretation is obtained by different sequences from different subspecies represent a change in the temperature in analysed-melting peak. The IIRM real-time PCR offers a new alternative for rapid, sensitive and subspecies specific for differentiation and detection of two main pathogenic *M. avium* subspecies in poultry and ruminant hosts. The high sensitivity and specificity suggest the present test will be of diagnostic value. The analytical sensitivity in this study was only 100 copies of Maa and Map DNA. Moreover, real-time PCR with HRM produced no cross reaction between Maa and Map or various DNAs from other bacterial species that usually infected duck and cow indicating high specificity.

There are several studies mention the application of IIRM real-time PCR for *mycobacterial*. Recently in 2017, the researchers defined comparison of melting curves of different non-tuberculosis mycobacteria and presented the melting curves corresponding to the 6 unique-specific HRM groups (*M. tuberculosis* complex, *M. kansasii*, *M. simiae*, *M. fortuitum*, *M. abscessus-M. chelonae* group, and *M. avium* complex) for the mycobacterial *rpoBC* locus in several collected clinical specimens [13].

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ACKNOWLEDGEMENTS

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REFERENCES

1. Thorel MF, Huchzermeyer H, Weiss R, Fontaine JJ. *Mycobacterium avium* infections in animals. Literature review. Vet Res. 1997; 28: 439-447. <https://bit.ly/2F3pMZ>
2. Thorel MF, Krichevsky M, Levy-Frebault VV. Numerical taxonomy of mycobactin-dependent mycobacteria, amended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *sibiricum* subsp. nov. Int J Syst Bacteriol. 1990; 40: 254-260. <https://bit.ly/2MzZD0v>

3. Dhama K, Mahendran M, Tiwari R, Singh SD, Kumar D, Singh S, et al. Tuberculosis in birds: Insights into the *Mycobacterium avium* infections. *Vet Med Int*. 2011; 2011: 712369. <https://bit.ly/2MDg2Br>
4. Ramirez J, Mason C, Ali J, Lopez FA. *Mycobacterium avium* complex pulmonary disease: management options in HIV-negative patient. *J La State Med Soc*. 2008; 160: 248-254. <https://bit.ly/2R4DUvq>
5. Mijs W, De Haas P, Rossau R, van Der Laan T, Rigouts L, Portaels F, et al. Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* for bird-type isolates and *M. avium* subsp. *hominissuis* for the human/porcine type of *M. avium*. *Int J Syst Evol Microbiol*. 2002; 52: 1505-1518. <https://bit.ly/2Z1ul5>
6. Rathnalah G, Zinniel DK, Bannantine JP, Stabel JR, Gröhn YT, Collins MT, et al. Pathogenesis, molecular genetics, and genomics of *Mycobacterium avium* subsp. *paratuberculosis*, the etiologic agent of Johne's disease. *Front Vet Sci*. 2017; 4: 187. <https://bit.ly/2ZgkTdh>
7. Thorel MF, Huchzemeyer H, Michel AL. *Mycobacterium avium* and *M. intracellulare* infection in mammals. *Rev Sci Tech*. 2001; 20: 204-218. <https://bit.ly/2WY39sl>
8. Reed GH, Kent JO, Wittwer CT. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics*. 2007; 8: 597-608. <https://bit.ly/2Ka90tG>
9. Tong SY, Giffard PM. Microbiological applications of high-resolution melting analysis. *J Clin Microbiol*. 2012; 50: 3418-3421. <https://bit.ly/2WrJXNJ>
10. Chamings A, Hewson KA, O'Rourke D, Ignjatovic J, Noormohammadi AH. High-resolution melt curve analysis to confirm the presence of co-circulating isolates of avian nephritis virus in commercial chicken flocks. *Avian Pathol*. 2015; 44: 443-451. <https://bit.ly/2XyQoPu>
11. Miller M, Zorn J, Briemeier M. High-resolution melting curve analysis for identification of *Pasteurellaceae* species in experimental animal facilities. *PLoS One*. 2015; 10: e0142560. <https://bit.ly/21G8ldP>
12. Santos GB, Espinola SM, Ferreira HB, Margis R, Zaha A. Rapid detection of *Echinococcus* species by a high-resolution melting (HRM) approach. *Parasit Vectors*. 2013; 6: 327. <https://bit.ly/217DLFO>
13. Khosrval AD, Hashemzadeh M, Hashemi-Shahraki A, Teimoori A. Differential identification of *Mycobacterial* subspecies using high-resolution melting analysis. *Front Microbiol*. 2017; 23: 2045. <https://bit.ly/2vOcWA2>
14. Issa R, Abdul H, Hashim SH, Seradja VH, Shañi N, Hassan NA. High resolution melting analysis for the differentiation of *Mycobacterium species*. *J Med Microbiol*. 2014; 63: 1284-1287. <https://bit.ly/2WBklZ2>
15. Castellanos E, Aranaz A, De Buck J. PCR amplification and high-resolution melting curve analysis as a rapid diagnostic method for genotyping members of the *Mycobacterium avium-intracellulare* complex. *Clin Microbiol Infect*. 2010; 16: 1658-1662. <https://bit.ly/2WIXI3>
16. Ricchi M, Barbieri G, Cammi G, Garbarino CA, Arrigoni N. High-resolution melting for analysis of short sequence repeats in *Mycobacterium avium* subsp. *paratuberculosis*. *FEMS Microbiol Lett*. 2011; 323: 151-154. <https://bit.ly/2R5dpXV>
17. Douarre PE, Cashman W, Buckley J, Coffey A, O'Mahony JM. High resolution melting PCR to differentiate *Mycobacterium avium* subsp. *paratuberculosis* cattle type and sheep type. *J Microbiol Methods*. 2012; 88: 172-174. <https://bit.ly/2R1VJML>
18. Castellanos E, Aranaz A, De Buck J. Rapid identification and differentiation of *Mycobacterium avium* subspecies *paratuberculosis* types by use of real-time PCR and high-resolution melt analysis of the MAP1506 locus. *J Clin Microbiol*. 2010; 48: 1474-1477. <https://bit.ly/2R3FCyh>
19. Galen RS. Predictive value and efficiency of laboratory testing. *Pediatr Clin North Am*. 1980; 27: 851-859. <https://bit.ly/2XJ5u56>

