Development and evaluation of semi-nested PCR for detection of the variable lipoprotein haemagglutinin (*vlhA*) gene of *Mycoplasma Synoviae* in chicken

Tawatchai Pohuang^{1,2}, Patchara Phuektes^{1,2}, Sucheewa Junnu^{1,2,*},

¹Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

²Research Group for Animal Health Technology, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

Abstract: This study aimed to develop a semi-nested polymerase chain reaction assay for the direct detection of *Mycoplasma synoviae* (*M. synoviae*) from clinical samples using three newly designed oligonucleotide primers specific to the variable lipoprotein haemagglutinin (*vlhA*) gene and differentiate *M. synoviae* field strains based on a nucleotide deletion or the insertion of the proline-rich repeat (PRR) region of the *vlhA* gene. The developed semi-nested polymerase chain reaction (PCR) assay revealed positive results in 12 out of 100 clinical samples collected from chickens showing lameness and joint swelling. Six positive samples were selected randomly for sequencing, and sequence analysis revealed 96.3-100% nucleotide identities compared to the reference sequences. Phylogenetic analysis showed that sequences of the strains in this study were closely related to WVU1853 (Spain), CK.MS.UDL.PK.2014.2 (Pakistan), and F10-2AS (USA) strains, but they were distinct from the *M. synoviae*-H vaccine strain sequence. *M. synoviae* obtained from these samples were identified as types A and C with a length of 38 and 32 amino acids, respectively. These results indicated that the specific and sensitive semi-nested PCR could be a useful diagnostic tool for the direct identification of clinical samples, and the sequence analysis of the partial *vlhA* gene can be useful for typing *M. Synoviae*.

Keywords: chicken, *Mycoplasma synoviae*, semi-nested PCR, *vlhA* gene

Introduction

Mycoplasma synoviae (*M. Synoviae*) is one of the most common *Mycoplasma* species causing infections in avian species worldwide [1]. *M. synoviae* causes a range of illnesses in infected birds, including chronic subclinical upper respiratory diseases, airsacculitis, eggshell apex abnormalities (EAA), and infectious synovitis and tenosynovitis [1,2]. These clinical signs adversely affect product performance and egg production and result in increased condemnation. Therefore, *M. synoviae* infections have caused numerous economic losses in the poultry industry throughout the world [3,4].

M. Synoviae infections can be transmitted vertically via eggs or horizontally via direct contact. Early detection of new infections and routine biosecurity monitoring is recommended for the elimination of *M. synoviae* transmission [4]. On the other hand, it is difficult to diagnose *M. synoviae* infections differentially from avian reovirus, *Staphylococcus aureus*, *Mycoplasma gallisepticum* (*M. Gallisepticum*), *Escherichia coli*, and *Pasteurella multocida* infections, based on the clinical signs [5]. Currently, the culture method is the gold standard for the identification of *M. synoviae* infection, but this method is laborious and time-consuming and can take up to 3-4 weeks to complete. The serological diagnosis of *M. synoviae* infection has several problems, including nonspecific reaction, cross-reactions with inactivated *M. gallisepticum* vaccination, and low sensitivity during the acute phase of infection [6,7]. A polymerase chain reaction (PCR) based on the 16S rRNA gene was developed for the rapid detection of *M. synoviae* infected flocks [8,9]. Moreover, other PCR-based assays, such as digitalized random amplified polymorphic DNA analysis and amplified fragment length polymorphism analysis, have also been used to genotype *M. Synoviae* [10]. With advantages over culture and serological methods, high sensitivity PCR assays, such as real-time PCR and nested PCR assays, have been used increasingly for the rapid, specific and sensitive detection, and identification of *M. synoviae* infections from

*Corresponding author

Sucheewa Junnu
Faculty of Veterinary Medicine, Khon Kaen University, 123, Mittraphap Frontage Rd., Khon Kaen 40002, Thailand

Tel: +66-86236-5459
Fax: +66-43-202-404
E-mail: sucheewa@kku.ac.th

ORCID

Tawatchai Pohuang
<https://orcid.org/0000-0002-4634-3995>
Patchara Phuektes
<https://orcid.org/0000-0001-5463-0284>
Sucheewa Junnu
<https://orcid.org/0000-0003-1081-7279>

Conflict of Interest
The authors declare no conflicts of interest.

Received: March 2, 2020
Revised: July 1, 2020
Accepted: July 9, 2020

clinical samples [3,4,11,12].

Although PCR assay targeting the 16S rRNA gene is now used routinely and extensively for the detection of avian *Mycoplasma* spp. infected flocks, the conserved nature of the 16S rRNA gene makes it unsuitable for *M. synoviae* strain differentiation by sequencing. Molecular detection and characterization of the variable lipoprotein hemagglutinin A (*vlhA*) gene has been used successfully for *M. synoviae* strain differentiation without the need for prior culture or isolation [13-15]. The *vlhA* gene of *M. synoviae* is cleaved post-translationally into an N-terminal lipoprotein (MSPB), which exhibits a high degree of antigenic variation [16]. *M. synoviae* strain typing based on nucleotide deletion or insertion within the proline-rich repeat (PRR) region of the *vlhA* gene is related to the invasiveness of *M. synoviae* associated with infectious synovitis [13]. This study aimed to develop a semi-nested PCR assay based on the *vlhA* gene for increased sensitivity of *M. synoviae* detection from clinical samples as well as to characterize the PRR region of the *vlhA* gene of *M. synoviae* for strain differentiation.

Materials and Methods

Positive and negative control DNA

M. synoviae DNA was kindly provided by the National Institute of Animal Health, Thailand, and the *M. synoviae*-H vaccine strain DNA positive control was derived from the modified-live vaccine Vanguard® plus 5/CV-L (Zoetis, A). The other pathogens, including the Newcastle disease virus (NDV), infectious laryngotracheitis virus (ILT), infectious bronchitis virus (IBV), and *M. Gallisepticum*, were local isolates and were proven by sequencing.

DNA preparation

The chromosomal DNA of *M. synoviae*-H and *M. Gallisepticum* vaccine strains, and DNA from clinical specimens were extracted using a previously published method [17] with some modifications. Briefly, DNA was extracted by placing swabs into 1 mL of PBS and twisting the swab vigorously. The suspension was then centrifuged at 13,000 rpm for 10 min. The pelleted cells were finally suspended in 50 µL of nuclease-free water to reduce the nuclease activity. The sample tubes were placed in a heating block at 100°C for 10 min to release DNA from the cells and were then refrigerated at -20°C for 10 min. After the cell lysate was centrifuged at 13,000 rpm for 10 min, the supernatant was collected and subjected to PCR. The viral nucleic acids of NDV, ILT, and IBV were extracted using the Viral Nucleic Acid Extraction Kit (Invitrogen, USA) according to the manufacturer's manual. The extracted nucleic acids were stored at -20°C until used. The concentration of *M. synoviae* DNA was measured using a spectrophotometer (BioDrop, UK) at the A260/A280 ratio, and the DNA was serially diluted ten-fold from 0.7 ng/µL for the semi-nested PCR assay sensitivity detection.

Primers selection and semi-nested PCR reactions

Three primers targeting the highly conserved region of the *M. synoviae vlhA* gene were newly designed based on an alignment of the *vlhA* genes available in the GenBank database. Based on the nucleotide sequence of the *M. synoviae* strain T68W/IT1A GenBank accession numbers GU451303.1, the forward primer position was at 288nt to 308nt, while an outer reverse primer and an inner reverse primer positions were at 988nt to 1009nt and 550nt to 571nt, respectively. The nucleotide sequences of the primers were as follows: MS-SCF1 (5'-GGT GAT CAA ACT CCA GCA CCT-3') used as the forward outer primer of the first-round PCR and a forward inner primer of the second-round PCR. MS-SCR2 (5'-TCC ACT TGC ATT TTT AGA CCA T-3') and MS-SCR1 (5'-GTT ACC GAT CCG CTT AAT GCT T-3') were used as a reverse outer primer of the first-round PCR and a reverse inner primer of the second-round PCR, respectively. The sizes of the PCR products of the first- and second-round PCRs were at 724-base pairs (bp) and 283-bp, respectively.

Optimization of the semi-nested PCR

All semi-nested PCR reactions were performed in a total volume of 25 µL. The first-round PCR mixtures were composed of 12.5 µL of the 2x DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, USA), 0.5 µL (25 µM) of MS-SCF1 and MS-SCR2 primer, 3 µL of DNA template, and 8.5 µL of RNase free water. The amplification steps were performed in a DNA thermal cycler (Major Science, USA) with initial heating at 94°C for 5 min and 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and polymerization at 72°C for 45 s, followed by a final elongation step of 10 min at 72°C. The second PCR reactions (nested-PCR) were composed of 12.5 µL of the 2x DreamTaq Green PCR Master Mix, 0.5 µL (25 µM) of MS-SCF1 and MS-SCR1, 3 µL from the first amplicons and 10.5 µL of RNase free water. The PCR amplification steps were conducted using the same conditions as described for the first-round PCR. Electrophoresis was performed to analyze the PCR products using 1.2% agarose gels containing 1X of GelRed™ Nucleic Acid Gel Stain (Biotium, USA). The gels were run at 100 volts for 30 min before visualization under ultraviolet light in the Gel Doc™ XR+ Gel Documentation System (Bio-Rad, USA).

Specificity test

The specificity of the primers developed in this study was tested against other pathogens that can cause respiratory diseases and infectious synovitis in chickens similar to *M. Synoviae*, including the Newcastle disease virus (NDV), infectious laryngotracheitis virus (ILT), infectious bronchitis virus (IBV), and *M. gallisepticum*.

Sensitivity test

A 0.7 ng/µL sample of purified *M. synoviae* DNA was serially diluted ten-fold for sensitivity testing, and the detec-

tion limits between the present semi-nested PCR and conventional PCR were compared [14]. The minimum concentrations showing positive results in both tests were noted.

Detection of *M. synoviae* DNA from lameness chickens

From June to August 2016, choanal and tracheal swabs were collected from 100 lame chickens raised in six commercial farms located in the eastern and western regions of Thailand. These chickens had joint problems, such as infectious synovitis and tenosynovitis. Their ages ranged from 36 to 42 weeks and 40 days for broiler breeders and broilers, respectively. The swabs were placed in phosphate-buffered saline (PBS) and kept at -80°C until used. The swab samples were submitted for DNA detection by a semi-nested PCR assay, as described above, and the presence of *M. synoviae* DNA was recorded.

Detection of anti- *M. synoviae* antibody from lameness chickens

All 100 serum samples collected from the lame chickens described above were examined for antibodies against *M. synoviae* using a commercial test kit, ProFLOK® (Synbiotics Corporation, USA) according to the manufacturer's recommendation. The diluted sera were added to *M. synoviae* anti-

gen-coated plates. The optical density was read using an ELISA reader at 405 nm and was calculated and interpreted according to the manufacturer's recommendation. Antibody titer of 0-269, 270-743, and higher than or equal to 744 were considered negative, suspect, and positive results, respectively.

Sequence analysis of partial *vlhA* gene of *M. synoviae*

Positive PCR samples were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany), and the purified DNA was subjected to DNA sequencing (First BASE Laboratories Sdn Bhd, Malaysia). DNA sequences of the PCR products were analyzed for sequence identity and similarity compared to positive *M. synoviae* and *M. Synoviae*-H. The BLAST program (www.ncbi.nlm.nih.gov/BLAST) and the BioEdit Sequence Alignment Editor Version 7.0.9.0 software were used for nucleotide sequence similarity analysis.

Phylogenetic tree analysis

The partial *vlhA* gene sequences identified were then analyzed by comparing with the sequences of other *M. synoviae* strains obtained from the GenBank database. The nucleotide (nt) and amino acid sequence alignment and relationship analysis were performed using MEGA6 software (<http://www.megasoftware.net>). Phylogenetic tree and distance anal-

Table 1. Historic details of the *M. synoviae* isolates used for sequence comparison and phylogenetic analysis

Isolate	GenBank accession number	Chicken type	Specimen	Age	Clinical signs	Location of isolation	Sample collection
KKUMSB1	KY930895	Broiler	Choanal swab	40 days	Joint swollen	Chonburi, Thailand	ELISA positive
KKUMSB7	KY930896	Broiler	Choanal swab	40 days	Joint swollen	Chonburi, Thailand	ELISA positive
KKUMSB9	KY930897	Broiler	Choanal swab	40 days	Joint swollen	Chonburi, Thailand	ELISA positive
KKUMSP3	KY930898	Broiler breeder	Choanal swab	36 weeks	Joint swollen	Chonburi, Thailand	ELISA positive
KKUMSP49	KY930899	Broiler breeder	Choanal swab	36 weeks	Joint swollen	Chonburi, Thailand	ELISA positive
KKUMSTF	KY930900	Broiler breeder	Choanal swab	42 weeks	Joint swollen	Kanchanaburi, Thailand	ELISA positive

Table 2. Reference *M. synoviae* strains used for sequence comparison and phylogenetic analysis

Strain	Type and subtype	GenBank accession number	Location	Additional information
WVU1853	A	AM998371	Spain	NA
K1968	B	KJ606929	USA	Turkey; meat turkey
MSH-Vaccine	C1	AB501271	Australia	Vaccine strain (Australia origin)
MSH	C1	KX168666	Thailand	Chicken
FMT	D	KC832825	USA	Chicken
F10-2AS	E1	HQ326482	USA	NA
AHRU2015CU2807.1	L	KX168688	Thailand	Chicken
AHRU2014CU5801.2	C1	KX168685	Thailand	Chicken
CHN-QZ114-1-2013	?	KU572389	China	Gallus gallus
JBSJ_Br	?	JQ684434	Malaysia	Broiler breeder chicken: choanal cleft and trachea
EAA	?	FJ495803	The Netherlands	NA
CBU080258	?	KM985996	South Korea	Layer chicken: tracheal swab
CK.MS.UDL.PK.2014.5	?	KP316021	Pakistan	Chicken: joints synovial fluid

NA, not available.

yses of the nucleotide sequences were constructed using MEGA6 software version 6.06 by Neighbor-joining with the maximum likelihood method based on the Kimura 2-parameter model with the neighbor-joining (NJ) method and 1,000 replications of bootstrap values [18]. Table 1 and Table 2 list the *M. synoviae* strains in this study and the reference *M. synoviae* strains, respectively.

Results

The expected PCR product sizes were 724 bp and 283 bp for the first and second rounds of semi-nested PCR, respectively. The primers designed in this study were based on the *vlhA* gene of *M. synoviae* accession number GU451303.1. The specificity of semi-nested PCR was tested against other pathogen species that commonly cause respiratory diseases in chickens. The results revealed the nonspecific bands of other pathogens DNA (Fig. 1). The semi-nested PCR sensitivity test results were 10^{-5} , 10^{-5} , and 10^{-9} for the conventional PCR, outer primers, and inner primers, respectively (Fig. 2).

Detection of *M. Synoviae* from clinical samples

One hundred choanal cleft swabs were detected for *M. synoviae* DNA using the semi-nested PCR. *M. synoviae* DNA was observed in 12 out of 100 swab samples (12%) tested, which gave 283 bp of *M. synoviae* specific bands by semi-nested PCR (Fig. 3).

Determination of *M. synoviae*-antibody

Six out of 100 serum samples had antibodies against *M. synoviae* and were positive for *M. synoviae* DNA by semi-nested PCR.

Sequence analysis of *M. synoviae* *vlhA* gene

The partial *vlhA* fragments of six randomly selected positive PCR samples were sequenced, and all six nucleotide sequences were submitted to GenBank (National Center for Biotechnology Information, Bethesda, MD). The GenBank

Fig. 1. Specificity determination: A total of 4 common respiratory pathogens were tested. M, molecular weight ladder; 1-2, infectious bronchitis virus; 3-4, infectious laryngotracheitis; 5-6, Newcastle disease virus; 7-8, *M. gallisepticum*; 9-10, negative control; and 11-12, positive control.

Fig. 2. Sensitivity detection of the conventional PCR (A), semi-nested PCR procedure using outer primers (B) and inner primers (C) detecting 10-fold dilution of the extracted genomic DNA of *M. synoviae*. M, molecular weight ladder; 1-9, 10-1-10-9 dilution of *M. synoviae* positive stock, respectively; NC, negative control; PCR, polymerase chain reaction.

Fig. 3. Polymerase chain reaction products from choanal and tracheal swabs for detection of *M. synoviae* in clinical samples, the sizes of 724 bp with the primers MS-SCF1&MS-SCR2 (outer primer) and 283 bp with the primers MS-SCF1&MSSCR1 (inner primer). M, molecular weight ladder; 1-12, DNA of clinical samples; NC, negative control; PC, positive control.

accession numbers were KY930895, KY930896, KY930897, KY930898, KY930899, KY930900 for KKUMSB1, KKUMSB7, KKUMSB9, KKUMSP3, KKUMSP49, and KKUMSTF strain, respectively. Sequence analysis showed that these six strains had 98.9 to 100% nucleotide sequence homology. The sequences of five strains, designated KKUMSB1, KKUMSB7, KKUMSB9, KKUMSP3, and KKUMSP49, had 89.5 to 100% homology with the reference strains and had complete identity with the WVU1853, F10-2AS and CK.MS.UDL.PK.2014.2 strains. The KKUMSTF strain of this study showed 90.7 to 98.9% homology with the sequences of the reference strains (Table 3).

A partial *vlhA* sequence alignment of 19 different strains revealed several nucleotide substitutions among these strains. The nucleotide sequence position 177 of five strains in this study (KKUMSB1, KKUMSB7, KKUMSB9, KKUMSP3, and KKUMSP49) was A (adenine) nt similar to the WVU1853, K1968, F10-2AS, and CK.MS.UDL.PK.2014.2 strains. In contrast, the other strains, including KKUMSTF, had G nt in this position. The nucleotide sequence position 225 of five strains (KKUMSB1, KKUMSB7, KKUMSB9, KKUMSP3, and KKUMSP49) was C (cytosine) nt, which was similar to the *M. synoviae*-H, F10-2AS, AHRU2014CU5801.2, and CK.MS.UDL.PK.2014.2 strains, whereas the KKUMSTF, K1968, FMT, AHRU2015CU2807.1, CHN-QZ114-1-2013, JBSJ_Br, EAA, and CBU080258 strains had T (thymine) nt. The nucleotide sequence position 238 of the KKUMSTF strain was G, which was similar to the reference strains, K1968, AHRU2015CU2807.1, CHN-QZ114-1-2013, JBSJ_Br, and CBU080258. The other reference strains and five strains of this study (KKUMSB1, KKUMSB7, KKUMSB9, KKUMSP3, and KKUMSP49) were T nt. Furthermore, the nucleotide sequence positions 308 and 336 of all six strains of this study were A and C similar to the WVU1853 and F10-2AS strains, while the other reference strains were C and

A nt, respectively (Fig. 4).

The deduced amino acid sequence analysis revealed 98.9 to 100% homology for all strains in this study, which was similar to the nucleotide sequence analysis. These six strains showed 93.9 to 100% sequence homology compared to the reference strains. The amino acid sequences of the five strains in this study (KKUMSB1, KKUMSB7, KKUMSB9, KKUMSP3, and KKUMSP49) showed 100% identity with the reference WVU1853 and CK.MS.UDL.PK.2014.2 strains (Table 3).

Fig. 5 Sucheewa Junnu

Fig. 5. Phylogenetic tree constructed by neighbor-joining method based on the partial sequence of *vlhA* gene of *M. synoviae*. The sequences were obtained from the 6 samples in this study (●), and 13 sequences from GenBank. Values at nodes indicate bootstrap probabilities, as determined for 1,000 re-samplings.

Fig. 4 Sucheewa Junnu

Fig. 4. Comparison of the deduced amino acid sequences of the 19 MS strains. The comparison was performed by the BioEdit software and the results are displayed graphic view. Symbols that are identical to the consensus are displayed in upper case. In comparison with the published sequence of the K1968 strain had an insertion of seven amino acids in the PRR (DNPQNPN) whereas the 5 strains (KKUMSB1, KKUMSB7, KKUMSB9, KKUMSP3 and KKUMSP49) are the WVU1853 strain. The KKUMSTF strain revealed deletions of 13 amino acids in the PRR region comparing with K1968 strain.

Table 3. Sequence homology of nucleotides and amino acids of *vlhA* gene in this study and reference strains

[illegible]

Based on the *vlhA* sequence analyses, the six *M. synoviae* field strains were identified as PRR type group A (KKUMSB1, KKUMSB7, KKUMSB9, KKUMSP3, and KKUMSP49) and C (KKUMSTF), with types A and C containing 38 and 32 amino acids, respectively (Fig. 4).

Phylogenetic analysis of the deduced amino acid sequences of the six strains and 13 reference strains showed that the present six strains belonged to two groups (Fig. 5). The five strains (KKUMSB1, KKUMSB7, KKUMSB9, KKUMSP3, and KKUMSP49) were allocated to the same group as the F10-2AS, CK.MS.UDL.PK.2014.2 and WVU1853 strains.

Discussion

Although conventional PCR has been used increasingly for *M. synoviae* detection over the past decade [14], few studies on nested PCR have been reported. Therefore, this semi-nested PCR assay may provide an alternative, sensitive, and useful diagnostic tool for *M. synoviae* detection. Semi-nested PCR was approximately 10,000 times more sensitive than conventional PCRs with the previously reported primers [14]. Therefore, semi-nested PCR enhances the sensitivity of *M. synoviae* determination. In addition, it was specific to *M. synoviae* detection, indicating that the primers were particular to *M. synoviae* nucleic acid. The DNA extracted from field samples collected from chickens showing clinical signs associated with *M. synoviae* infections was tested directly by the semi-nested PCR, and positive results were observed.

Moreover, the infection rate, according to the ELISA results, revealed lower sensitivity than the direct detection of swab samples using semi-nested PCR. On the other hand, *M. synoviae* infection diagnosis and monitoring should use ELISA for disease confirmation and screening the status of chicken flocks. In addition, partial *vlhA* gene amplification is useful for *M. synoviae* strain identification [19]. The primers specific to the *vlhA* gene had been designed [13,14,20], in which the resulting PCR products could be analyzed further by sequencing, and the genotype of *M. Synoviae* could be identified.

Several reports on molecular and evolutionary analysis of *M. synoviae* strains based on the *vlhA* gene have been published [2,13,15,21-23,28]. Sequence alignment and phylogenetic analysis based on the partial *vlhA* gene of this study demonstrated that the field strains were different from other *M. synoviae* strains and the live commercial vaccine strain that has been reported earlier [21,22,24,25]. Phylogenetic analysis indicated that the KKUMSB1, KKUMSB7, KKUMSB9, KKUMSP3, and KKUMSP49 were closely related to the WVU1853, CK.MS.UDL.PK.2014.2, and F10-2AS strains isolated from Spain, Pakistan, and the USA, respectively. Based on *vlhA* gene sequencing and analysis, Ogino et al. [22] identified the vaccine strain MS-H and field strains rapidly without any cultures or isolation methods. Similar to previous studies [14,15,22], the sequence analysis results of this study could differentiate the field strains from the *M.*

synoviae-H strain based on the *vlhA* gene, without prior cultures. In addition, the six strains obtained from lame chickens in this study displayed a high identity of their nucleotide and amino acid sequences. Sequencing analysis results revealed sequence homologies indicating that these strains were closely related to the WVU1853, F10-2AS, and CK.MS.UDL.PK.2014.2 strains.

Currently, eleven types of *M. synoviae*, type A-K, have been classified based on the insertion or deletion of the PRR region [13,14,23,26]. In Thailand, previous studies on *M. synoviae* differentiation based on partial *vlhA* gene sequence analysis reported at least three types, including types C, E, and L. Type C and E were obtained from chickens with respiratory signs and type L were obtained from chickens showing clinical lameness and joint swelling without respiratory signs [7]. The current *M. synoviae* field strains could be classified as types A and C similar to the reference WVU1853 (type A) and MS-H (type C) strains [14]. These strains were detected and identified from choanal cleft swabs collected from chickens showing clinical joint swelling without respiratory signs, indicating that the developed semi-nested PCR was useful for identifying and screening *M. synoviae* infections in commercial chicken farms. Although the KKUMSTF strain was classified as type C (32 AAs) similar to the MS-H strain, the sequences of the deduced amino acids were a different deletion and insertion compared to the reference strain, K1968. This result indicated that KKUMSTF could be classified further into subtypes [13,18]. In addition, this type was associated with infectious synovitis, with the chickens showing clinical lameness and joint swelling without respiratory signs similar to the previous report of Thai *M. synoviae* isolates type L [27]. The six clinical samples found to be PCR-positive but did not present the antibody reactor detected by ELISA may indicate an early MS infection. This finding is similar to a previous study showing that the PCR procedure could first detect the MS DNA, but did not reveal the antibody reactor detected by either the SPA test or ELISA or show any clinical signs [29].

In conclusion, the specific and sensitive semi-nested PCR was developed based on the *vlhA* gene for *M. Synoviae* detection and identification. This PCR enables the rapid detection of *M. Synoviae* and molecular characterization of *M. synoviae* directly from clinical samples that may be useful for a further study of *M. synoviae* evolution and differentiation.

Acknowledgements

This study was supported by the Research Fund of the Faculty of Veterinary Medicine, Khon Kaen University, Thailand and Research Group for Animal Health Technology, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, Thailand. The author wishes to thank Dr. Patcharee Thongkamkoon and Zoetis (Thailand) Limited for supporting this research.

References

- Kleven SH. *Mycoplasma synoviae* infection. In: Calnek BW, Barnes HJ, Beard CW, McDougald LR, Saif YM (eds.). *Diseases of Poultry*. 11th ed. pp. 220-228, Iowa State Univ. Press, Ames, IA, 1997.
- Feberwee A, de Wit JJ, Landman WJ. Induction of eggshell apex abnormalities by *Mycoplasma synoviae*: field and experimental studies. *Avian Pathol* 2009;38:77-85.
- Kleven SH. Control of avian mycoplasma infections in commercial poultry. *Avian Dis* 2008;52:367-374.
- Landman WJ. Is *Mycoplasma synoviae* outrunning *Mycoplasma gallisepticum*? A viewpoint from the Netherlands. *Avian Pathol* 2014;43:2-8.
- Tawfik RG, Khalil SA, Ellakany HF, Torky HA. *Mycoplasma synoviae* and other associated bacteria causing arthritis in chicken. *Alex J Vet Sci* 2016;49:163-169.
- Ewing ML, Cookson KC, Phillips RA, Turner KR, Kleven SH. Experimental infection and transmissibility of *Mycoplasma synoviae* with delayed serologic response in chickens. *Avian Dis* 1998;42:230-238.
- Kleven SH, Rowland GN, Kumar MC. Poor serologic response to upper respiratory infection with *Mycoplasma synoviae* in turkeys. *Avian Dis* 2001;45:719-723.
- Lauerman LH, Hoerr FJ, Sharpton AR, Shah SM, van Santen VL. Development and application of a polymerase chain reaction assay for *Mycoplasma synoviae*. *Avian Dis* 1993;37:829-834.
- Uddin MI, Abid MH, Islam MS, Rakib TM, Sen AB, Chowdhury SM, Anwar MN, Kamaruddin KM. Molecular identification of *Mycoplasma synoviae* from seroprevalent commercial breeder farms at Chittagong district, Bangladesh. *Vet World* 2016;9:1063-1069.
- Feberwee A, Dijkstra JR, von Banniseht-Wysmuller TE, Gielkens AL, Wagenaar JA. Genotyping of *Mycoplasma gallisepticum* and *M. synoviae* by amplified fragment length polymorphism (AFLP) analysis and digitalized random amplified polymorphic DNA (RAPD) analysis. *Vet Microbiol* 2005;111:125-131.
- Hess M, Neubauer C, Hackl R. Interlaboratory comparison of ability to detect nucleic acid of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by polymerase chain reaction. *Avian Pathol* 2007;36:127-133.
- Huang L, Xie Z, Xie L, Deng X, Xie Z, Luo S, Huang J, Zeng T, Feng J. A duplex real-time PCR assay for the detection and quantification of avian reovirus and *Mycoplasma synoviae*. *Virol J* 2015;12:22.
- Benčina D, Drobnič-Valić M, Horvat S, Narat M, Kleven SH, Dovc P. Molecular basis of the length variation in the N-terminal part of *Mycoplasma synoviae* hemagglutinin. *FEMS Microbiol Lett* 2001;203:115-123.
- Hong Y, García M, Leiting V, Benčina D, Dufour-Zavala L, Zavala G, Kleven SH. Specific detection and typing of *Mycoplasma synoviae* strains in poultry with PCR and DNA sequence analysis targeting the hemagglutinin encoding gene *vlhA*. *Avian Dis* 2004;48:606-616.
- Jeffery N, Gasser RB, Steer PA, Noormohammadi AH. Classification of *Mycoplasma synoviae* strains using single-strand conformation polymorphism and high-resolution melting curve analysis of the *vlhA* gene single-copy region. *Microbiology (Reading)* 2007;153:2679-2688.
- Noormohammadi AH, Markham PF, Whithear KG, Walker ID, Gurevich VA, Ley DH, Browning GF. *Mycoplasma synoviae* has two distinct phase-variable major membrane antigens, one of which is a putative hemagglutinin. *Infect Immun* 1997;65:2542-2547.
- Lauerman LH. *Mycoplasma* PCR assays. In: Lauerman LH, Turlock CA (eds.). *Nucleic and Amplification Assays for Diagnosis of Animal Diseases*. pp. 41-42, American Association of Veterinary Laboratory Diagnosticians, Visalia, CA, 1998.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 2013;30:2725-2729.
- Dijkman R, Feberwee A, Landman WJ. Variable lipoprotein haemagglutinin (*vlhA*) gene sequence typing of mainly Dutch *Mycoplasma synoviae* isolates: comparison with *vlhA* sequences from Genbank and with amplified fragment length polymorphism analysis. *Avian Pathol* 2014;43:465-472.
- Wetzel AN, Lefevre KM, Raviv Z. Revised *Mycoplasma synoviae vlhA* PCRs. *Avian Dis* 2010;54:1292-1297.
- Harada K, Kijima-Tanaka M, Uchiyama M, Yamamoto T, Oishi K, Arao M, Takahashi T. Molecular typing of Japanese field isolates and live commercial vaccine strain of *Mycoplasma synoviae* using improved pulsed-field gel electrophoresis and *vlhA* gene sequencing. *Avian Dis* 2009;53:538-543.
- Ogino S, Munakata Y, Ohashi S, Fukui M, Sakamoto H, Sekiya Y, Noormohammadi AH, Morrow CJ. Genotyping of Japanese field isolates of *Mycoplasma synoviae* and rapid molecular differentiation from the MS-H vaccine strain. *Avian Dis* 2011;55:187-194.
- Bayatzadeh MA, Pourbakhsh SA, Ashtari A, Abtin AR, Abdoshah M. Molecular typing of Iranian field isolates *Mycoplasma synoviae* and their differentiation from the live commercial vaccine strain MS-H using *vlhA* gene. *Br Poult Sci* 2014;55:148-156.
- Pourbakhsh SA, Maghami M, Ashtari A, Bayatzadeh MA, Ahangaran S. The *vlhA* gene sequencing of Iranian *Mycoplasma synoviae* isolates. *Arch Razi Inst* 2013;68:117-124.
- Ghaniei A. Molecular characterization of *Mycoplasma synoviae* isolated from broiler chickens of West Azarbaijan province by PCR of *vlhA* gene. *Vet Res Forum* 2016;7:197-202.
- Hammond PP, Ramírez AS, Morrow CJ, Bradbury JM. Development and evaluation of an improved diagnostic PCR for *Mycoplasma synoviae* using primers located in the haemagglutinin encoding gene *vlhA* and its value for strain typing. *Vet Microbiol* 2009;136:61-68.
- Limpavithayakul K, Sasipreeyajan J, Pakpinyo S. Characterization of Thai *Mycoplasma synoviae* isolates by sequence analysis of partial *vlhA* gene. *Avian Dis* 2016;60:810-816.
- Sun S, Lin X, Liu J, Tian Z, Chen F, Cao Y, Qin J, Luo T. Phylogenetic and pathogenic analysis of *Mycoplasma synoviae* isolated from native chicken breeds in China. *Poult Sci* 2017;96:2057-2063.
- Pakpinyo S, Khanda S, Boonyapisitsopa S. Surveillance of *Mycoplasma synoviae* infection in mixed Thai native chickens in the area of Nakornpathom Province. *Wet Chasan Sattawaphaet* 2009;39:267-273.