Investigation of Avian Mycoplasma Infection in Vietnam by Molecular Tools

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Summary

Poultry production plays an importance role in Vietnamese animal production. The main system for raising poultry in Vietnam is mainly based on low-input low-output system in small household. Such systems are currently bringing a certainly economic importance in poultry production but they are characterized by high incidence of disease. The Chronic Respiratory Disease and synovitis are caused by *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) in chicken and turkey are the diseases that causes a tremendous economic loss in Vietnam. The control of these diseases depends largely on early detection and eradication. Recently, a number of molecular diagnostic methods were developed in our institute in order to increase the sensitivity of mycoplasma detection. A nested PCR for the detection of *MG,* the main pathogen, was set up. The test reaches the limit of less than 5 CFUs/reaction, a multiplex PCR protocol and a PCR-RFLP procedure were set up for avian mycoplasma detection and strains differentiate with high sensitivity and accuracy. Our results shown that there are clear difference of MG infection between two seasons of study (86.8% in spring and 27.3% in autumn). There is a significant difference between two systems of poultry production in spring, the large scale (75%) and extensive production (87%). Interestingly, all of the pathogen avian mycoplasmas were found in Vietnam. The method enable us to detect the bacteria not only in swab sample but also allow us examine in other specimen such as yolk, embryo, water, litter…

Introduction

Avian mycoplasmosis can cause considerable economic losses in poultry industry, especially on chicken and turkeys in all over the world. MS causes respiratory disorder and synovitis in chicken, turkeys and other avian species, especially when MS infection is combined with Newcastle disease, infectious bronchitis. MS cause significant economic impact on chicken broilers, drop in egg production[18],[8]. MS infection occur mainly in chicken and turkeys. However, it has also been found in guinea fowl, ducks, geese, pigeons, quail, pheasants, house sparrows[18]. This infection is widely spread and many reports on the increasing of MS pathogenic. For veterinary medicine, four species MG, MS, MM, and MI are in great concern. MG is responsible for chronic respiratory disease (CRD) in chicken, infectious sinusitis in turkeys, it causes reduction in weight gain, decrease in food conversion efficiency and meat quality, increases mortality rate in broilers. In breeders and layers, the diseases cause a tremendous drop in egg production, increase in embryo mortality, leading to infected eggs and infected progeny flocks. MG also causes respiratory disease, infectious sinusitis and conjunctivitis in other avian species as pheasants and partridges [2] in songbirds [10], quail, ducks, geese [13].

Recently, a number of efforts were made to develop molecular methods for the detection of avian mycoplasmas within clinical specimens. The SDS-PAGE method (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used to strains differentiate based on protein profiles[12].The RFLP method (Restriction Fragment Length Polymorphism) based on patterns of DNA digested by various restriction enzymes on gel electrophoresis to identify different species and strains. DNA probes with radioactively or non-radioactively labeled is used for mycoplasma detection but the sensitivity is low. The sensitivity will be improved when combining DNA probes with PCR methods[5],[6]. The most useful method for mycoplasma

detection is PCR. Some PCR procedures with high sensitivity have been published for MG and also for some other pathogen mycoplasmas[10],[9],[13],[18],[1]. Multiplex PCR, PCR-RFLP also developed for mycoplasmas detection and identification[7]. PCR with arbitrary primers and RAPD (Random Amplify Polymorphic DNA analysis)[10][2][4] are used for strains differentiation and very useful for epidemic study of these diseases.

In Vietnam, most of the poultry farms are equipped with poor condition and do not use any kind of vaccine against mycoplasmosis. Mycoplasmas infection is a big problem in many farms but it was considered less important. Researches on this disease are limited and rely on studying the infected situation by RSA test screening, clinical signs, lesions observation and setting up antimicrobial therapies in Vietnam condition. Diagnostic of this disease based mainly on clinical signs and sometimes on rapid serum agglutination test (RSA). PCR technique for M.gallisepticum diagnosis have been applying in Molecular Genetic Laboratory –National Institute of Animal Husbandry (NIAH) since 1998. Isolation of avian mycoplasmas have been carried out in National Veterinary Institute and National Center for Veterinary Diagnostic but little success was gained.

The present study describes the nested PCR techniques were used to detect M.gallisepticum in clinical samples. Different methods for sample treatment has been considered to eliminate the inhibitor agents. Nested –PCR protocols and results were compared with single-PCR and estimated of sensitivity by colony forming unit (CFU).

Materials and methods

Mycoplasma strains.

Typed trains of *M.gallisepticum* (PG31), *M.synoviae* (WVU1653), *M.iowae* (695), *M.gallinarum* (PG16), *M.imitans* (4229), *A.laidlawii* (PG8) and clinical samples were cultivated in modified Hayflick's-medium (B-medium), Frey-Medium and SP4 medium according to IOE [20], [18]. The CFU estimation was following John Lindquist et al.[21].

DNA extraction.

For broth culture, the heat-shock method was used (1 ml sample was centrifuged, the supernatant was discarded, the pellets was washed with 100µl of water, then samples were incubated in 95oC for 10 minutes, put in ice in 10 minutes. DNA extraction using cetyl-trimethylammonium bromide (CTAB) according to Maass and Dalhoff[23].

For clinical samples (throat sampling), two methods were applied:

a) Sampling by cotton buds, stored in PBS buffer at 4oC overnight; DNA extraction (Silveria et al. [17].

b) Sampling by cotton buds, stored in modified Hayflick medium[32]. Incubation for 24 h at 37oC and process to DNA extraction by heat-shock methods.

Primers selection

The nested PCR using two sets of primer to amplify the sequence of interest. The first set (Mgal1 and Mgal2) had been published as a specific primers for M.gallisepticum [7] with sequences and location on the 16s rRNA MG gene as in the fig.1. The second set of primer was designed based on the first primer information such as G+C contain, melting temperature, primer length with the aid of DNAsis software(HITACHI). The sequence and location of the 2nd set of nested PCR primer(FP5/RP5) was showed in fig 1.

Mycoplasma gallisepticum strain 16S ribosomal RNA gene, partial sequence.
ACCESSION No M22441

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   1 ttttctgaga gtttgatcct ggctcaggat taacgctggc ggcatgccta atacatgcaa
  61 gtcgatcgga tgtagcaata cattagaggc gaacgggtga gtaacacgta tccaatctgc
 121 cttatagtgg gggataacta gtcgaaagat tagctaatac cgcataacaa gttaactatc
 181 gcatgagaat aactttaaag aagcaactgc ttcgctataa gatgagggtg cggcatatca
 241 gctagttggt gagggtaatg gcccaccaag gcgatgacgt gtagttatgc tgagaggtag
 301 aataaccaca atgggactga gacacggccc atactcctac gggaggcagc agtagggaat  FP5
 361 ttttcacaat ggacgaaagt ctgatggagc aatgccgcgt gaacgatgaa ggtcttttta
 421 gattgtaaag ttcttttatt tgggaagaac agttagtaga gtggaaagct attaatttga
 481 ctgtaccatt tgaataagta acgactaact atgtgccagc agtcgcggta atacataggt
 541 tgcaagcgtt atccggattt attgggcgta aaacaagcgc aggcggatta gaaagtctgg
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 661 gagagtcctg gaactccatg tggagcggtg aaatgcgtag atatatggaa gaacaccaga  Mgal1
 721 ggcgaaggcg aggacttggg ccaatactga cgcttaggct tgaaagtgtg gggagcaaat
 781 aggattagat accctagtag tccacactgt aaacgatgga tgttaagtgt cggagcgaat
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 901 aaactcaaac ggaattgacg gggacccgca caagtggtgg agcatgttgc ttaattcgac  RP5
 961 ggtacacgaa aaaccttacc tagacttgac atcttgggcg aagctataga aatatagtgg
1021 aggtcaaccc aatgacaggt ggtgcatggt tgtcgtcagc tcgtgtcgtg agatgttggg
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1261 aaagttggtc tcagttcgga ttgagggctg caattcgccc tcatgaagtc ggaatcacta
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1381 tcaaactatg agagctggta atatctaaaa ccgtgttgct aaccgcaagg aagcgcatgt
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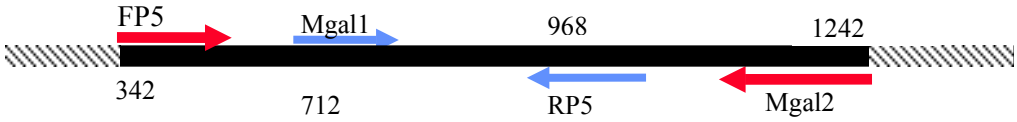

Fig. 1. Schematic map of Nested PCR on 16s rRNA small subunit gene

The primers combination was FP5/Mgal1 for first amplification (901 bp product) and RP5/Mgal2 for nested PCR (256 bp product).

PCR protocols.

First PCR amplification was used outer primers (FP5/Mgal2) to amplify the sequence from bp No342 to bp No 1242, the expected product is 901 bps with the PCR condition as follows:

After denaturation at 95°C for 5 minute, the first reaction was performed in 25 cycles with denaturation (95°C/40''), annealing (55°C/1′) and extension (72°C/1'20''). The reaction component for 25 µl include: 10 mM Tris HCl(pH 8.8), 50 mM KCl, 1,5 mM MgCl2, each deoxynucleoside triphosphate at a concentration of 200 µM, 2 pmole of Mgal2 and FP5 primers, 1 UI TaqDNA polimerase (Promega) and 2 ml of DNA samples.

For the 2nd PCR reaction, two primer sets Mgal1 and Mgal2 or Mgal2 and RP2 can be used. The former gives 525 bp product and the later gives 256 bp product. We had tested both set of primer on several mycoplasma strains and Mgal2 and RP2 have been shown to be appropriate for 2nd PCR test.

The components in the 2nd PCR are the same with the 1st reaction except the DNA sample is 1 µl of first PCR product and the amount of nested primers are 10 pmole(Mgal1,RP2).

The amplification condition is: First step is 94°C for 3 min; 2nd step is 94°C for 40"; 62°C for 50''; 72°C for 30'' with 25 cycles and final step is 72° C /5 min.

The PCR products and 100 bps markers were subjected to electrophoresis at 100 V in horizontal gels containing 1.5% agarose with 1xTBE buffer. The gel was stained with ethidium bromide (0.5 µg/ml), exposed to u.v light to visualized the results, and photographed with Pharmacia Image Master system.

Result and discussion

Nested PCR establishment

A new set of primer was designed and complemented on the 16s rRNA smallsub unit gene. The reaction conditions were optimized to successfully detect the presence of MG from referent strains of mycoplasmas and E.coli. The positive reactions were observed on *M.gallisepticum* and *M.imitans*. This result was agreed with the sequencing analysis and results reported by Kiss et al. 1997 [7], Nascimento et al. 1995[13]. This can be explained buy the highly homogenous of two species. Marois et al. 2001[12] reported that the homology of two species are 60 % and 53% when using PFGE (pulsed-field gel electrophoresis) and RADP (random amplified polymorphic DNA), respectively. The homology of two sequence of 16s RNA genes is 99% and can differentiate by using restriction enzymes. *M.imitans* is the pathogenic mycoplasma of duck and goose and used to be considered as a train of *M.gallisepticum*.

The sensitivities testing

Referent strain of MG (or MS) in the log phase cultivation broth medium was used. A serial dilution of 1/10 (or 1/5) was made, each dilution was made by adding 100 µl of sample to 900 µl of broth medium. 50µl of each dilution was inoculated to agar plate and incubated in 5%CO_2 incubator for 5 -7days. The numbers of colonies in the countable plates (plate with 30-70 colonies is preferred) were counted. The CFUs per ml from undiluted samples was calculated.

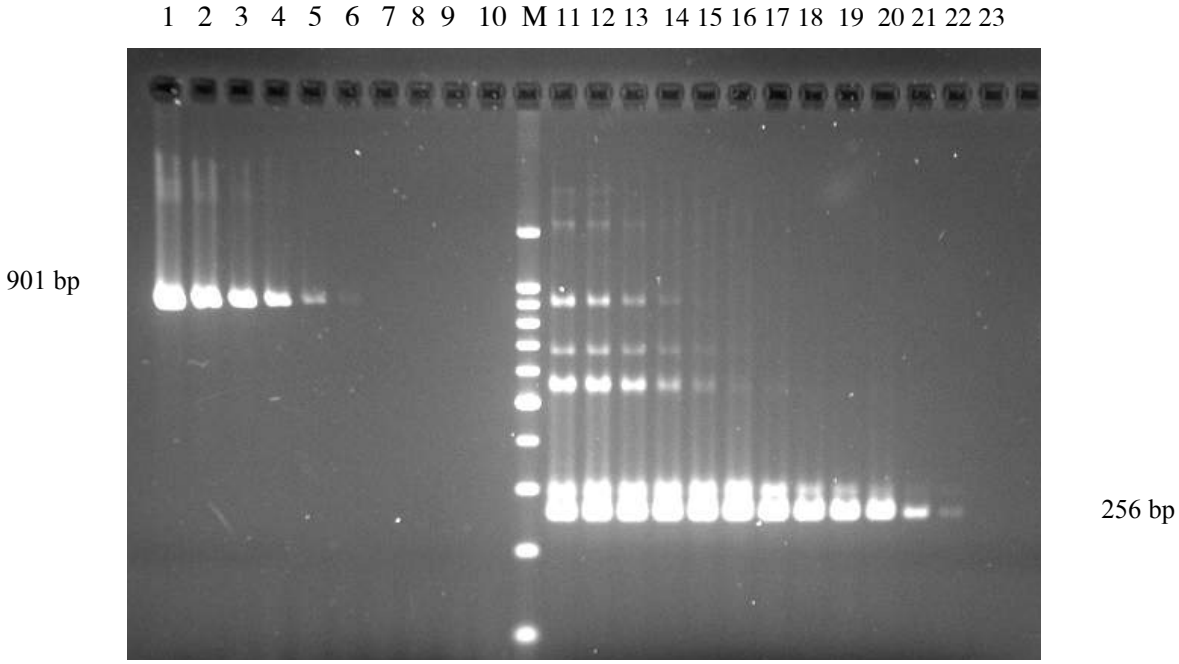

Fig 2. Sensitivity of one-step PCR and Nested PCR for MG detection.
Lane 1-10: first PCR products 901 bp. Lane M: Molecular weight standard Lane 11-23: nested –PCR product 256 bp

The first step PCR gives positive result in the 6th dilution meanwhile the nested PCR can give positive at the 11^{th} dilution. With starting concentration of $1.3x10^9$ CFUs/ml, 10 times dilution, 200 µl was used for DNA extraction, DNA was suspended in 20µl of water and 2µl was used for 25µl PCR reaction. The sensitivity of the first step PCR is $2.6x10^2$ CFU per reaction. For nested PCR, the reaction gave positive at the 11^{th} dilution, the sensitivity is less than 1 CFU per reaction ($2.6 x10^{-3}$ CFU). This result may be puzzling to non-mycoplasmologists, it is a well-known fact that 1 CCU or 1CFU corresponds to approximately 100-1000 mycoplasma cells due to the clump

tendency of mycoplasma cells during their development. More over. a large number of invisible cells and unable-to-grow cells due to a number of reason during the incubation. The MG genome may contain 3 regions of 16srRNA have 3 copies [16] thus the chance of primer annealing and amplification will increasing.

Nested PCR for the detection of MG on clinical samples

219 clinical samples were collected in two seasons, the weather conditions in northern province of Vietnam have tremendous effects to animal production, animal health and disease prevalence. The samples were different in management condition (intensive and scavenging models). Samples were extracted and analyzed by nested PCR method. Results were shown in fig3 and table 1.

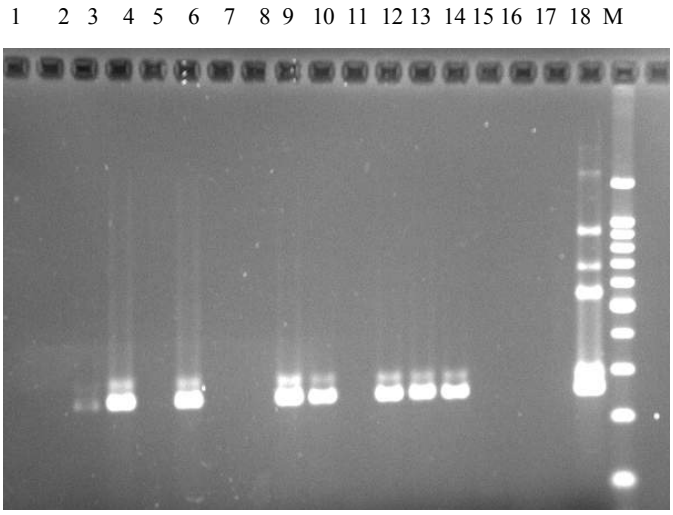

Fig 3. Nested PCR for *M.gallisepticum* detection on clinical samples.

Lane 1-17: clinical samples.

Lane 18: Positive control

Lane M: molecular weigh standard

Table 1. The infection result on chicken herds – by season.

Detection methods	**Nested PCR**		**Single PCR**	
Season	**positive**	**%**	**Positive**	**%**
Spring (197 samples)	171	86.8	112	56.8
Autumn (183 samples)	50	27.3	22	12.0
Totals (380samples)	***221***	***58.1***	***134***	***35.2***

The result shown that the sensitivity of two methods of detection is significant different ($p<0.01$), on the average the nested PCR give higher positive results thus it can be considered that the nested PCR is more sensitive than single PCR. Almost all the positive samples in single PCR or multiplex PCR are positive in the nested PCR

The differences in positive ratio between two seasons can be explained by the differences in climate condition. It is wet and cold in spring and most of the farms were equipped with inadequate condition, the resistance ability of bird is decreased and it is the favorable condition for mycoplasmas to spread among the flocks.

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