Molecular Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* Infection in Poultry

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Abstract

In Pakistan, production of poultry has been evolved as a good alternate of beef and mutton. In this study multiplex PCR approaches were adopted to differentiate and detect avian mycoplasma in a single PCR reaction as a step forward in the economization of PCR detection. From the total of 25 samples, 25 (100%) had a positive *Mycoplasma* isolation result. The biochemical test yielded the highest number of isolations, with MG accounting for 10/25. Commercial DNA-based PCR kits have been developed as a result of recent advancements in diagnostic techniques for *Mycoplasma* infections. Tetracyclines, fluoroquinolones, tilmicosin, tylosin, spiramycin Infections should be reduced as much as possible.

Keywords: Molecular detection • *Mycoplasma gallisepticum* • *Mycoplasma synoviae* • Poultry

Introduction

In Pakistan production of poultry has been evolved as a good alternate of beef and mutton. Government is facilitating the investors to develop it at an accelerate pace by virtue of which it has become the second largest business in Pakistan [1]. According to the economic survey of Pakistan commercially poultry production estimated in 2006-2007 was 401.0 million per day 23.8 million layer birds, 315.8 million broiler birds, 7.1 million breeding stock, and 5222.0 million eggs [2]. Commercially 480.0 tons production of poultry meat was estimates poultry production in rural areas in million No's was 38.0 day-old chicks, 37 layers-8 layers' birds, and 13.1 cocks and cockerels, Agriculture Economics Survey, Government of Pakistan, 2006-2007 [3]. The avian Mycoplasmosis is diagnosed by serological and isolation, while its time consuming and difficult process [4]. The improvement of the diagnosis of the infection of avian *Mycoplasma* has been recently developed by PCR. The duplex PCR assay targeting the heme–agglutinin multigenes families e.g vlhA for *Mycoplasma synoviae* and pMGA for *Mycoplasma gallisepticum* [5]. In this study multiplex PCR approaches were adopted to differentiate and detect avian mycoplasma in a single PCR reaction as a step forward in the economization of PCR detection [6].

The important avian pathogens are *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) and cause poultry loses [7]. Molecular biology techniques are currently used for a rapid detection of these pathogens and the adoption of control measures of the diseases [3].

Methodology

25 sick bird samples were collected from infected farm environment with the symptoms of respiratory disorder's including air sacs exudates, trachea, and oral swabs.

Culturing of samples

In the brain heart infusion samples were inoculated directly and incubated for 6 days-14 days at 37°C. The infusion colour changes was indication of positive reaction and the color of phenol change red to yellow (Table 1).

Table 1. Composition of Brain heart infusion.

Ingredients	Quantity
Difco (bacto-brain heart infusion)	3.7 g
dH_2O	90 ml
Horse serum	10 ml
Thallium acetate (1%)	1.0 ml
L-cysteine hydrochloride (10%)	1.5 ml
Phenol red (10%)	1.5 ml

Isolates identification

The isolation was identified and analyzed by serological, chemical, biochemical, and morphological parameters under light microscope using the low magnification (4 X and 10 X) colony were observed on plates by applying low intensity light. Biochemical tests The biochemical tests were designed to detect the specific enzymatic and nutritional activity of Mycoplasmas that was relatively small.

Glucose fermentation test

The Brain heart infusion broth containing 10% inactivated horse serum, 1% glucose, and 0.005% phenol red. Dispense aseptically in 5 ml amount into the tube, 0.1 ml diluted culture added into the test tube.

Phosphate production

The inoculated test culture plates containing 0.1% sodium phenolphthalein di-phosphate and plates were incubated at 37°C. the plates were flooded with 5 M NaOH after the growth of mycoplasma. The reaction positive indication through color changes, after 30 s the pink color appear in the colonies area.

DNA extraction

Extraction of DNA from culture and s swab samples were conducted through non-phenolic method which involve the step of boiling, freezing and centrifugation, the supernatant used as a reaction mixture.

PCR

The conditions for the multiplex PCR reaction were followed. The primer pairs sequence used for the PCR protocol is shown in Table 2.

Table 2. Forward and reverse primer sequence of M. *gallisepticum*.

Primers	Sequence	Base pair
MG Forward primer	5'CTTTCCCATCTCGACCAGGAGAAA-3'	732bp
MG reverse primer	3'GGATCAATCAGTGAGTAACGATGA-5'	732bp

Each PCR mixture contained 5 µl of 500 mMol KCL, 5 µl of 100 mMol

Tris HCL, 4 µl of 2.5 mMol MgCl, 2 µl (each) DATP, DCTP, DGTP, DTTP, 1 µl of each primer, 2.5 U of amplified *Taq DNA polymerase*, 62.5 µl of ultra pure water and template Mycoplasma DNA in 5 µl volumes were then added to a mixture. All of the DNA amplifications were carried out in the thermal cycler (model Peqlab 2 s primus, Germany). The optimum conditions were programmed in thermal cycler.

Electrophoresis

Amplified product of multiplex PCR were electrophoresis on the 1.5% agarose gel and visualize under gel documentation system.

Results

Out of the 25, 10 samples were of trachea, 6 of air sac, 4 of oral swab and 5 of lungs tissues. These characterize on the basis of biochemical and cultural studies. The biochemical profile of *Mycoplasma gallisepticum* including glucose fermentation and phosphatase activity were given in Table 3. Optimization of multiplex polymerase chain reaction was done by varying the annealing temperature varying the MgCl concentrations in the reaction mixture and varying the concentration of template DNA. Firstly, optimizing of annealing temperature of the thermal cycler ranging from 48°C to 58°C and its effect on the amplicon was noted. In electrophoresis the intensity of the band revelead its significance. At 48°C, no amplification was observed, at 50°C amplification was observed in *Mycoplasma gallisepticum*. DNA amplified well. At 54°C *Mycoplasma gallisepticum* DNA amplified very well. The results are shown in Table 4.

Secondly, the effect of decreasing and increasing the concentration of MgCl was observed and optimized at better result. A total of 25 samples were processed through multiplex polymerase chain reaction. These samples were categorized into 5 groups including 5 samples each. The concentration of MgCl lies between 1.5 mMol gave 2^+ results, 2.5 mMol gave 3^+ results and 3.0 mMol gave better results. From the above data it was concluded that the concentration of 2.5 mMol gave better results and the concentration of better results of MgCl was optimized at 2. 5 m Mol. Thirdly, optimizing of multiplex PCR was carried out by using different amount of template DNA or target DNA after visualizing in gel documentation system. Three different quantities of 5 µl, 8 µl and 10 µl of the template DNA were used and the band intensity was noted. Table 5 is showing the results for this parameter.

Table 3. Biochemical profile of *Mycoplasma gallisepticum*.

Species	Glucose fermentation	Phosphatase activity
Mycoplasma gallisepticum	+	-

The positive (+) and negative (-) sign show the band intensity.

Table 4. Effect of change in the annealing temperature on the amplification reaction mixture for optimization of multiplex PCR condition.

Serial no.	Mycoplasma spp.	Annealing temperatures			
		48°C	50°C	52°C	54°C
1	*Mycoplasma gallisepticum*	-	++	+	+++

At 48°C band intensity was negative (-), no bands will appear.

At 50°C band intensity was intermediate (++).

At 52°C band intensity was very low (+).

At 54°C band intensity was very high (+++).

Table 5. Effect of template DNA quantity on the reaction mixture for multiplex PCR test condition.

Serial no.	Mycoplasma spp.	Template DNA	Band intensity
1	MG	5 µl	+
2	MG	8 µl	+++
3	MG	10 µl	++
4	MG	12 µl	+

MG=*Mycoplasma gallisepticum*

Band intensity was very low (+).
Band intensity was intermediate (++).
Band intensity was very high (+++).

Discussion

In Pakistan, poultry has emerged as a viable alternative to beef and mutton. A drop in egg production of 10%-20%, an increase in embryo mortality and chick mortality of 5%-10%, and a fall in weight gain and feed conversion efficiency of 10%-20% are all losses caused by *Mycoplasma* infection. Only four of the 22 identified species recovered from avian sources were established pathogens for domestic poultry, namely *Mycoplasma gallisepticum*. Chronic Respiratory Condition (CRD) is the name for the disease caused by *Mycoplasma*. *Mycoplasma gallisepticum* is the most important pathogenic and commercial pathogen, and the disease caused by it has been declared as notifiable by the Office International des Epizooties (OIE). It is a big concern in the poultry business around the world, because sinusitis causes infection in turkeys. Losses related to Mycoplasmosis, specifically *MG* infection, include decreased egg productivity and egg quality, poor hatchability (high rate of embryonic mortality and culling of day-old birds), low feed efficiency, increased mortality, and carcass condemnation, in addition to medicine costs [8].

Various specimen samples were collected from clinically chronic respiratory disease cases of broiler and layer chickens, including trachea, air sac swab, oral swab, and lungs tissues, for the successful isolation of Mycoplasma species, but the trachea and air sac samples were the sample of choice because it is the predilection site of manipulation of *Mycoplasma* species. Beef heart infusion, peptones, yeast extract, and serum are the most important nutrients for the growth of *mycoplasma* spp. Along with NAD and cysteine, serum is a critical need for *Mycoplasma* growth. Additionally, because *Mycoplasma* are absolute resistant to these medications, penicillin and relative insusceptible thallium acetate are utilized to produce selective [9]. *Mycoplasma* species obtained in Brain Heart Infusion broth had a morphology that was tiny, smooth, circular, and had a fried egg look with a central opaque and outer translucent area. Several researchers have observed similar traits in *mycoplasma* species colonies. From the total of 25 samples, 25 (100%) had a positive *Mycoplasma* isolation result. The biochemical test yielded the highest number of isolations, with MG accounting for 10/25. Commercial DNA-based PCR kits have been developed as a result of recent advancements in diagnostic techniques for *Mycoplasma* infections. However, the reaction cost for clinical samples increased by a factor of two. The multi-species PCR allowed for the detection of multiple *Mycoplasma* in a single sample. The methods for detecting pathogenic avian mycoplasma using multiplex PCR described above are specific, sensitive, and consistent. It assisted us in enhancing the specific and accurate diagnosis of Mycoplasmosis in our commercial poultry sector by employing specific primer sequences and contributed to the elimination and reduction of losses due to Mycoplasmosis [10].

Conclusion

Tetracyclines, fluoroquinolones, tilmicosin, tylosin, spiramycin Infections should be reduced as much as possible. *MG* infection status is crucial for business in birds, hatching eggs, and chicks because eradication of this virus has been a primary goal of official poultry health programmes in most countries. These programmes rely on uninfected chick purchases, all-in/all-out production, biosecurity, and regular serological monitoring. Preventative medication of known infectious flocks may be beneficial in some cases. In Chickens & Turkeys: Ruffled feathers, fluid in the eyes (little bubbles in the corners of the eyes), cough, sniffing, breathing, bubbling, bruised face, strained crow in cockerels, drop in laying in hens, poor appetite, sweet smelling breath.

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