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Comparison of Conventional Bacterial isolation, Rapid Slide Agglutination and Polymerase Chain Reaction for Detection of *Mycoplasma gallisepticum* in Breeder Flocks

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Abstract

During *Mycoplasma gallisepticum* (MG) infections, the sensitivity of diagnostic tests and rapid diagnosis of infectious agent is particularly important. Utilizing of just one diagnostic technique such as isolation identification or serological tests can lead to the false results. This study was aimed to investigate different methods like isolation identification, rapid serum agglutination (RSA) test and polymerase chain reaction (PCR) for detection of MG infected poultry breeder flocks. A total 1255 serum samples and 1324 tracheal swabs were collected from 53 breeder poultry farms. By isolation and identification method 5/53 (9%) breeder farms were found infected with MG. The results of RSA test from the sera samples exhibited 9/53 (17%) positive flocks. PCR assay indicated 14/53 (26%) positive flocks. Based on the results of these studies, PCR is useful method for early diagnosis of MG outbreaks

Key words: *Mycoplasma gallisepticum,* Breeder flocks, PCR.

Introduction

Chronic respiratory disease is caused by *Mycoplasma gallisepticum* (MG) in chickens, resulting in reduced feed conversion egg production and significant downgrading of carcasses at slaughter. Transmission can occur through eggs or by inhalation of contaminated airborne droplets, resulting in rapid disease transmission throughout the flock. MG is a highly infectious respiratory pathogen affecting poultry. The clinical signs associated with MG infection in chickens include respiratory rales, nasal discharge, coughing, and occasionally conjunctivitis (Saif at al., 2003). Programs for control and eradication of the pathogen from breeder flocks are traditionally based on serological testing and isolation of the organism. However, it is difficult to diagnose MG infections in poultry flocks on the basis of clinical signs, routine culture procedures and commonly used serology (Mallinson, 1983; Yoder, 1986). The diagnosis of MG infection traditionally has been done by serology. Some of the disadvantages of serological methods are false-positive and false-negative reactions due to interspecies cross-reactions and nonspecific reactions. Moreover, there are some *Mycoplasma* species, e.g. *Mycoplasma iowae* showing antigenic heterogeneity and poor immune response that makes the development of reliable serological methods for detection more difficult (Jefferey *et al*., 1995). In vitro isolation of the organisms is usually used to confirm serological results. However, confirmation of an isolate by growth inhibition requires considerably additional time and monospecific antisera which are expensive. These techniques are time consuming, labor intensive and there are chances for false negative and false positive results. (Fan *et al*., 1995)

Recently, the rapidly developing nucleic acid-based molecular biological techniques have been employed and PCR based methods have proved to be excellent tools for Rapid and effective identification of *Mycoplasma* strains. (Han Wang *et al.,* 1997, Feberwee *et al.,* 2005, Ben *et al*., 2005).

In this study, we compare the investigations from different breeder flocks by rapid serum agglutination (RSA) test, Isolation and identification of the MG and the PCR based assay for the detection of MG.

Material and Method

Sample collection

A total no of 1255 serum samples and 1324 tracheal swabs were collected from a total no of 53 breeder farms located at Sehala and Tret area of Islamabad having a population of 150,000 breeder flocks for detection and prevalence of MG.

Collection of Serum Samples

Blood from breeder birds were collected aseptically in sterile 5ml syringes and allowed to clot in an

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upright position. After 4 hours the syringes were placed in refrigerator at 4°C. After 24 hours clear serum were collected from syringes and centrifuged at 3000 x *g* for 5 minutes to clear from debris. The clear serum were collected in appendrof tubes, labeled and stored at -20°C for further studies.

Collection of Tracheal Swabs

Sterile cotton swabs with wooden applicators were used to collect tracheal swabs for isolation of MG and PCR analysis. The swabs were dipped in mycoplasma broth before the specimens were taken (Zain & Bradbury 1996). and then replaced in the swab holders for transportation under chilling conditions.

Isolation of *Mycoplasma gallisepticum*

Pleuropneumonia-like organism (PPLO) broth and agar was prepared in two parts

Part A Pleuropneumonia-like organism (PPLO) broth base without crystal violet (Difco) (14.7 g); distilled or deionised water (700 ml).

Part B: Horse serum (heated at 56°C for 1 hour) (150 ml): 25% (w/v) fresh yeast extract (100 ml); 10% (w/v) glucose solution (10 ml); 5% (w/v) thallous acetate (10 ml); 200,000 International Units (IU)/ml penicillin G (5 ml); and 0.1% (w/v) phenol red solution (20 ml). The pH was adjusted to 7.8.

Part A was autoclaved and after cooling, it was added to Part B that was sterilized by filtration.

For the solid medium, PPLO broth was replaced by PPLO agar. The mixture was autoclaved as before and kept in a water bath at 56°C. The constituents of part B were mixed separately and then incubated at 56°C. Parts A and B were mixed dispensed into Petri dishes.

For isolation of MG tracheal swabs were inoculated into PPLO broth. Cultures were examined daily for the evidence of growth for 7 to 10 days. Fermented broth medium (change in color from red to yellow) was transferred to fresh media for adaptation and then streaking was performed on solid media plates. Agar plates were examined for typical fried egg shaped mycoplasma colonies. Mycoplasma strains were identified by morphological and cultural means followed by biochemical tests (fermentation of glucose, tetrazolium and failure to hydrolyse arginine).

Rapid serum agglutination test

Rapid serum agglutination (RSA) test was performed by mixing equal amount (20 µl each) of test serum and antigen (Intervet International) at room temperature on thoroughly cleaned white tile and rotated for three minutes. All agglutination reactions were scored from 0 to 4 (0 being no agglutination and 4 being complete agglutination). All serum samples yielding agglutination reactions (2+ or greater) were subjected to serial two fold dilution (1:4 to 1:8) made in phosphate buffer saline and retested.

Polymerase chain reaction

Deoxyribose nucleic acid (DNA) was extracted from swab samples suspended in 3 ml of PPLO broth in a sample collection tube (Lauerman, 1998). The 1.5ml broth was poured in eppendrof tube and centrifuged for 30 minutes at 14,000 x ***g*** at 4°C. The supernatant was removed and the pellet was suspended in 25 µl PCR-grade water. The tube and the contents were boiled for 10 minutes and then placed on ice for 10 minutes before centrifugation at 14,000 x ***g*** for 5 minutes. The supernatant containing DNA was used as template in PCR reaction. For the detection of MG species-specific primer pairs were used. The sequence of the forward primer MG-14F: 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3' the sequence of the reverse primer was: MG-13R: 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3' (Lauerman, 1998).

The amplification reaction was performed in a total volume of 50 µl containing 5 µl of 10X PCR buffer (Fermentas), 250 µM concentrations of each deoxynucleotide triphosphate (Fermentas), 50 pmol of each forward and reverse primers, 2.25 mM $MgCl_2$, 2 U of *Taq* DNA polymerase (Fermentas) and 10 µl of DNA template. DNA amplifications were performed in a thermal cycler (Thermo USA) that was programmed to heat the DNA at 94°C for 5 min, followed by 35 cycles at three different temperatures and times (94°C for 1min , 55°C for 1min s and 72°C for 1 min) that corresponded to target DNA denaturation, primer annealing and primer extension. The final extension step was 72°C for10 min and soak at 4°C.

PCR products of 185bp were detected by conventional agarose (1.5%) gel electrophoresis, 50bp ledder (Fermantas) was used for accurately determining the size of PCR product, followed by viewing under UV light and photographed.

Data analysis

The numbers of positive results according to the different tests were analyzed using the one way ANOVA. A significance level of 5% was used.

Results

The serum and tracheal swabs samples were collected for rapid slide agglutination Test and for isolation of bacteria by conventional streak plate method and By PCR. For this purpose the swabs collected from different birds were immediately placed into collection vessel containing PPLO broth and chilled at ice for transportation. After transportation half of the liquid containing swab was shifted into PPLO broth and half of the material was subjected to centrifugation for isolation of DNA.

Tracheal swab collected from different birds was subject to isolation and identification of MG both by conventional streak plate method and PCR analysis.

A total no of 123 samples showed growth by conventional streak plate method after 5-10th day of streaking. While a total no of 155 samples showed positive agglutination with RSA test and total no of 206 samples were positive by PCR analysis as shown in table 1.

By isolation and identification method 5/53 (9 %) breeder form were found infected with MG. The results of RSA test from the sera samples exhibited 9/53 (17%) positive flocks. PCR assay indicated 14/53 (26%) positive flocks (Figure 1). MG Primers used in the study successfully amplified 185 base pairs amplicon in MG positive samples were as no band was seen in reaction where *E. coli* DNA was used as negative control (Figure 2)

PCR test significantly detected more positive birds as compared with RSA tests and isolation studies. The numbers of positive results for isolation studies were significantly different from RSA test

Table 1: Isolation, PCR and serology results for detection of *Mycoplasma gallisepticum* (MG) infected birds in poultry Breeder flocks

Tests performed on samples	No. of positive swab samples	%age of positive swab samples	No. of positive serum samples	%age of positive serum samples
Isolation of MG	123	9%	-	-
PCR	206	16%	-	-
RSA	-	-	155	12%
Total No. of swab samples	1324	`	-	-
Total No. of serum samples	-	-	1255	-

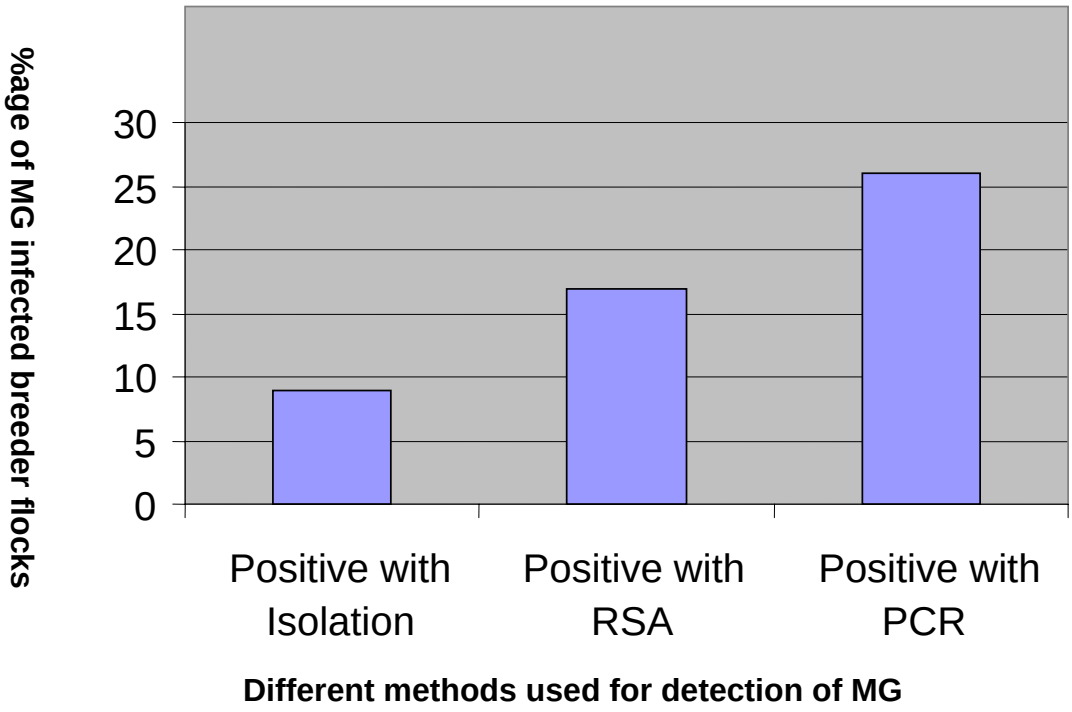

Fig 1: Comparison of different techniques for detection of *Mycoplasma Gallisepticum* (MG) infected breeder Flocks

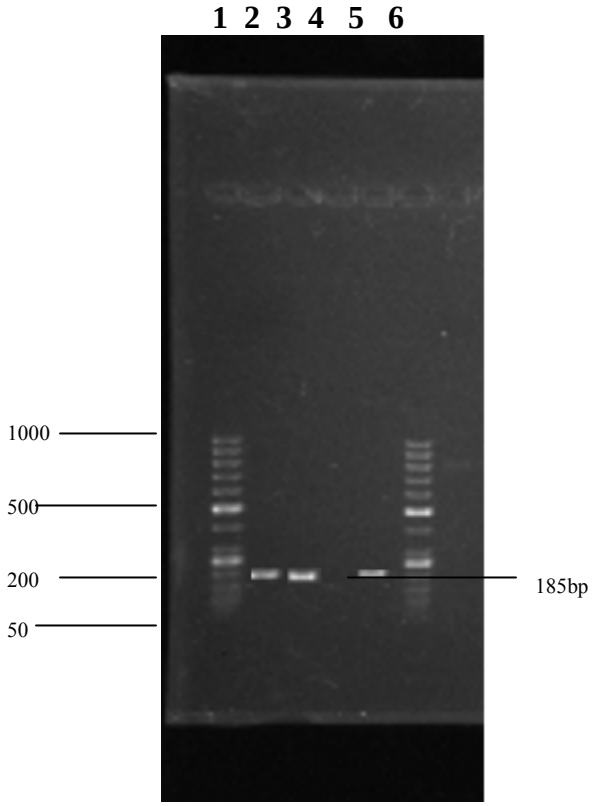

Fig 2: PCR product of 185bp: Lane 1,6 50bp ladder, Lane 2, 3,5 185bp product, Lane 4 *E. coli* DNA as negative control.

Discussion

The detection of MG infection usually depends on isolation by culturing on media and conventional serological tests. In this study, according to the culture results, 5/53 breeder flocks were positive. When compared with results from study by Dingfelder *et al.,* (1991) the percentage of positive swabs appear rather low. It can be speculated, that under field conditions, when only a small percentage of chickens are actually contaminated, or when birds harbor other mycoplasmas or bacteria that hamper the isolation of MG, the cultural confirmation of such infections may be very difficult to achieve and may necessitate a high number of samples or alternative methods. In the present study, RSA tests with commercial antigens revealed more positive results (9/53) as compared to the isolation and identification. The most widely used serological test for MG monitoring is the rapid slide agglutination test. According to Roberts (1969), chickens infected with three different strains of MG always reacted serologically with the homotypic and the heterotypic RSA antigens. Thus the numbers of positive or suspicious chickens according to the two tests were significantly different. A similar result was reported by Lin and Kleven (1982) with strains K503 and K730 which were shown to differ serologically from classic MS strains such as A5969. In the RSA test, birds singly infected with the variant strain had high antibody titres against the homologous antigen and a variable but lower response against the other antigens. It is well established that antigen differences between the hemagglutinin of the field strain and the diagnostic strains may lead to false negative results. For mycoplasmas, the development of diagnostic tools is difficult due to the problem of antigenic proteins undergoing high frequency variation in typical strains as Levisohn *et al* (1995) established that surface antigens of MG are subjected in vivo to rapid alteration in their expression. This variability may function as a crucial adaptative mechanism, enabling the organism to escape from the host immune defense and to adapt to the changing host environment at different stages of a natural

infection. Thus, diagnostic tools should be able to cope with a wide spectrum of antigen presentations.

PCR based methods are sensitive and used successfully for the detection of mycoplasm species. A 185 base pairs (bp) amplicon appeared in mycoplasma positive flocks by PCR. Similarly, 732, 1900 and 500bp products were amplified for detection of MG in laboratory and clinical isolates (Ben *et al.,* 2005, Han Wang *et al.,* 1997). Sensitivity of the PCR method was highest as compared to isolation and RSA test Detection limit. Ewing *et al*., 1996 reported that the RSA and HI tests failed to detect early *Mycoplasma synoviae* infection in newly introduced flocks while ELISA and PCR detected new infections on these farms and these results are in line with our studies (14/53). Feberwee *et al.* (2005) compared two commercially available PCR tests, rapid plate agglutination (RPA) test, hemagglutination inhibition (HI) test, and eight commercially available enzyme-linked immunosorbent assays. The results of the serologic tests showed that a certain level of false-positive results can be expected in about any serologic test. PCR test was found more sensitive. Although the level of false-positive results varied between several serologic tests, Detection for MG positive samples is highest by PCR assay as compared to RSA and isolation. This study depicts the superiority of PCR techniques on serological methods; however it is advisable to apply different test systems for final conclusion.

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