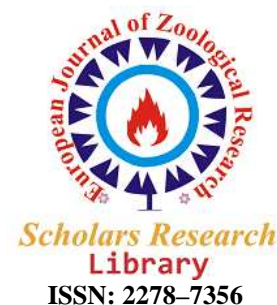




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Evaluation of Mycoplasma Gallisepticum Infection diagnosis in rural poultry by 16S rRNA PCR methods

Kamrouz Kaboli¹, Peyman Bijanzad^{2*}, Amid Reza Jeyrani Moggadam², Morteza Shahbazi² and Hossein Hosseini³

¹Young Researchers and Elite Club, Tabriz Branch, Islamic Azad University, Tabriz, Iran

²Department of Clinical Sciences, Tabriz Branch, Islamic Azad University, Tabriz, Iran

³Department of Clinical Sciences, Karaj Branch, Islamic Azad University, Karaj, Iran

ABSTRACT

Mycoplasma gallisepticum (MG) is one of the most important diseases in Iranian poultry industry and all over the world. Mortality, poor weight gain and increasing of feed conversion ratio (FCR) were seen in MG infected flocks, and causes economical losses particularly in broiler chickens that were used for meat production. In this study, rural broiler breeder farms located around east azarbaijan of Iran were investigated during 2011-2012. In each farm 18 to 31 chickens were sampled. The prevalence of Mycoplasma gallisepticum was studied by ELISA test, and then all positive cases were further examined by 16s rRNA methods. Our results indicated that the from 73 samples were tested, 23 (31.50%) of samples were positive in ELISA. All seropositive samples were positive in PCR. It can be concluded that ELISA serological tests should be only used as screening in monitoring programs to detect MG in poultry flocks and positive results should be confirmed by routine microbiological tests. The higher rate of MG in rural broiler breeder flocks indicated that these farms do not consider biosecurity and hygienic conditions.

Key words: Mycoplasma Gallisepticum, RSA, ELISA, Serological methods, Broiler breeder.

INTRODUCTION

Mycoplasma gallisepticum (MG) is one of the most important disease in poultry production and also it is the causative agent of chronic respiratory disease in chickens (14). MG infection causes significant economic losses in the poultry industry due to downgrading of carcasses at slaughter because of airsacculitis, treatment costs, and due to its effect on flocks performance (19), and reduction of egg production in chickens, turkeys and other avian species were reported (18). MG infection mainly is transmitted through ovaries, and the MG-infected breeder flocks should be depopulated; hence, the preferred method for MG control is to maintain MG-free flocks (29). However, in some situations such as multi-age production farms, maintaining the flocks free of MG may be difficult or impossible. Also MG infection is of high economic significance because of high morbidity and high mortality.

MG can be diagnosed by its different properties such as microbial culture, biochemical and serological properties (12, 18). Serology is the only reliable tools for detecting the subclinical infection in the flock (1). There are two major Serological methods, which were used for screening breeder farms in Iran, Rapid Serum Plate Agglutination (RSA), and Enzyme Linked Immunosorbent Assay (ELISA); however, there were differences in sensitivity and specificity of these methods.

Eradication is the most important control measure for MG infections in poultry production. Especially eradication of vertically transmitted agents, early detection of new infections is extremely important. For a long period, control and

prevention programs were based on use of the rapid serum plate agglutination (RSA) test, Hemagglutination inhibition (HI) test, and culture. Recently, enzyme-linked immunosorbent assays (ELISAs) have been introduced.

There were some difficulties in use of serologic tests for *Mycoplasma* that has been described previously. Problems with the use of the RSA test, particularly when undiluted sera are tested, are: a) nonspecific reactions due to bad quality or freezing of the sera, b) properties of the antigen preparation, c) recent use of inactivated vaccines, and d) cross-reactions based on the antigenic relationship between MG, *Mycoplasma Synoviae*, and *Mycoplasma imitans* (5, 10, 25, 26, 30). However, lack of specificity and/or sensitivity of ELISAs in the acute phase of infection has been reported (2,3,30,47). ELISA has been proved to have good sensitivity and more specificity compared to RSA (16). In addition, the isolation procedure may not always be successful alone due to the overgrowth of nonpathogenic mycoplasmas in culture (31). To solve these problems, Species specific recombinant DNA probes for the diagnosis of MG have been developed (13). Despite excellent specificity of these probes, it is important that at least 10^6 organisms were needed to detect by this method (31) and thus MG DNA probes may not be able to detect organisms from specimens taken during late stages of infection. In order to developing sensitive diagnostic methods we used the polymerase chain reaction (PCR) (21, 22) and primers designed on the basis of the 16S rRNA genes (17) for detection of MG from field samples.

The aim of the present study was to diagnosis of *Mycoplasma gallisepticum* in rural breeder farms by 16S rRNA, method.

MATERIALS AND METHODS

In this study, broiler breeder rural farms located in east azarbaijan province of Iran were Selected and investigated during 2011-2012. From each flock 18 to 31 chickens (a total of 73 samples) were randomly selected for blood collection, 2 ml of blood was collected aseptically from wing vein of each bird and then sera were separated and stored at -21°C until use for ELISA tests. The antibody against MG detected in serum from each flocks with commercial ELISA kit (IDEXX, USA). The procedure was followed according to the manufacturer instruction.

Trachea and choanal cleft samples from live birds were collected using sterile cotton swabs and were each inoculated into 3 ml broth media (*Mycoplasma* broth base, Frey, BBL, US) containing 15% inactivated horse serum. Inoculated broth media were shipped by overnight carrier to laboratory and incubated at 37°C as soon as possible. Samples were observed for the evidence of growth and kept in incubator for one month before being discarded as negative culture. Any suspected growth was subcultured on Frey's media agar and incubated in a very moist atmosphere containing 5% CO_2 (15). The presence of MG was confirmed with PCR amplification of a segment in 16S rRNA specific for *Mycoplasma gallisepticum* as described later (17).

DNA extraction, A 2-ml quantity of broth culture at lag phase was used for DNA extraction. The suspension was centrifuged at $13,000 \times g$ for 30 min at 4°C , washed two times with phosphate-buffered saline (PBS), and the pellet was resuspended in 25 μl PBS. The samples were boiled for 10 min, cooled on ice for 10 min, and centrifuged at $13,000 \times g$ for 5 min. The supernatant was removed and stored at 4°C (17, 20).

Amplification of rRNA, The primers for amplification of 16S rRNA were MG-14F (5'-GAG CTA ATC TGTAAG GTT GGT C-3') and MG-13R (5'-GCT TCC TTG CGG TTA GCA AC-3') as described earlier (17). The amplification was carried out in 50 μl reaction volume consisting of 5 μl 10 x PCR buffer, 1 μl 10 mM dNTP, 0.5 μl of each primer (20 μM), 0.25 μl Taq DNA polymerase (5U/ μl), 2 μl 50 mM MgCl_2 , 39.75 μl of deionized distilled water and 1 μl of template DNA. All amplification reaction were performed in a Gradient Mastercycler (Eppendorff, Germany) as follows: 94°C for 3 min, followed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 5 min.

Gel electrophoresis, The PCR products were detected by gel electrophoresis (Apelex, France) in 2% agarose (Agarose MP, Roche) gel in TAE buffer. Gels were run for 1.5 hr at 60 V, stained with ethidium bromide, destained with distilled water, exposed to ultraviolet light and photographed (Visi-Doc-It system, UVP, UK). Commercial DNA ladders were used as molecular-weight markers in each gel running.

RESULTS AND DISCUSSION

Mycoplasma infections are important poultry disease that causes economical losses in poultry production. Purpose of this study was to investigate of antibodies against *Mycoplasma gallisepticum* and also detection of MG in seropositive chickens.

The results of the serologic tests demonstrated that a certain level of false positive results can be expected in any test. Although the level of false-positive results varied between several serologic tests, for this reason it is not advisable only to rely completely on one test (6). All MG diagnostic tests (especially serology) showed a lower sensitivity in the detection of infection with some MG and MS strains (6).

16S rRNA. Amplification of samples from seropositive chickens with diagnostic primers MG-14F/ MG-13R yielded a PCR product of 185 base pairs (bp) from all MG strains and isolates (Figure 1).

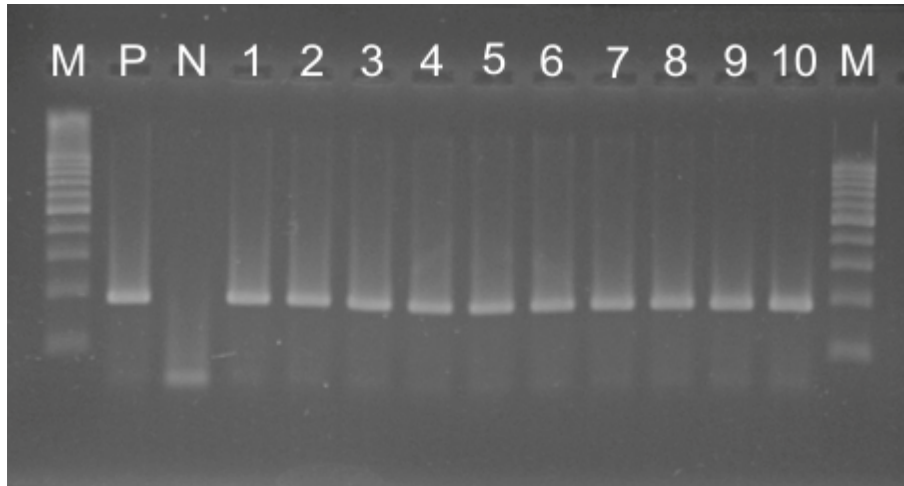


Figure 1: Amplification of 16S rRNA. M: Ruler 100bp DNA ladder. P: Positive control, N: Negative Control, Lane 1 to 10 field isolates

Serological test results indicated the frequency of antibodies against MG detected by ELISA was 31.50% (23/73). All seropositive samples examine by 16S rRNA PCR, and results indicated that all of them were positive in PCR test

The OIE recommends the use of serological tests for avian mycoplasmosis only as screening tools in the diagnosis of flocks, not of individual birds. This recommendation is based on the presupposition that tests have different sensitivities and specificities (6, 23). Also researchers indicated that the screening programs that are only based on seroconversion may be inadequate for mycoplasmosis diagnosis and control (4).

However, atypical infections with low immunogenic potential may cause false negative results. Some studies suggested the isolation methods should be used only in case of positive serological results (8). In addition, the type of antibody detected by serological tests varies, while RSA detects IgM antibody found 3 to 5 days after infection, and which persists for 70–80 days, but the HI and ELISA tests detect IgG antibody found 7 to 10 after infection, and which persists for up to 6 months (3).

Our results indicated that *Mycoplasma gallisepticum* infection in rural broiler breeders is prevalent Although the results of our study in agreement with previous studies, but due to controlling rules that approved by Iranian Veterinary Organization, MG positive parent flocks were slaughtered and the higher rate of MG in rural broiler breeder flocks indicated that these farms do not consider biosecurity and hygienic conditions. Additionally, it was proved that the occurrence of *Mycoplasma gallisepticum* have a relationship with the sampling year, season and ages of chickens, which should be studied more in detail.

High prevalence rate of MG infection was reported previously by several studies in poultry farms (11, 24, 27). Some researchers mentioned that the seroprevalence of MG infection was higher (33.3%) in female than in male (10.14%), which it is indicating that the female birds significantly ($p < 0.05$) were more susceptible than male birds. Isolation and identification of MG in Ghaemshahr town in north of Iran, showed that 20% of broiler farms positive in case of Mycoplasma genus and 12 percent of farms positive in molecular tests. Also several researches was indicated that, regardless of the screening of broiler breeder farms and control of MG, still high prevalence of MG present in poultry farms of Iran (7, 9). Previous studies on broiler breeder farms in Iran indicated high seroprevalence (21.4%) of MG (2, 28). Also it was reported that the prevalence of MG infection was higher (56.21%) in female than in male (43.79%) (7, 28).

However, intensive nature of poultry farming provided opportunity for recycling of the pathogens due to population density. The other factors that contribute MG infection are poor ventilation, contamination of litters and no

restriction on the movement of the technical personnel, visitors and such other persons as well as other biosecurity measures (24).

CONCLUSION

These serological methods should be only used as screening tests in monitoring programs to detect avian mycoplasmosis in poultry flocks and positive results should be confirmed by routine microbiological tests. Differences in the results of the serological tests confirm this information, that the use of other techniques necessary to confirm the presence of the MG, such as culture and/or DNA detection by molecular assays (PCR).

Although the RSA and ELISA test was described to be less sensitive than the HI test, but it has the advantage of being rapid and easily performed and therefore can be utilized as a routine flock test. Our results indicated that all positive ELISA cases were positive in PCR assay but more studies should be conducted. Also it is distinct that the molecular methods more sensitive and specific in diagnosis of MG and the molecular methods could determine the organism and use of this method causes quick detection of disease in flocks.

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