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Detection of *Mycoplasma gallisepticum* in Chaharmahal Va Bakhtiari Province Poultry Using PCR

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Abstract: Mycoplasmas are very small bacteria lacking cell walls that belong to various genera within the class Mollicutes and also the smallest organisms that can live independently. They are able to cause serious and chronic disease because of some unique characteristics. *Mycoplasma gallisepticum* is an important avian pathogen causing significant economic losses within the poultry industry. The aim of the present study was to investigate the prevalence of *M. gallisepticum* in poultry in Chaharmahal Va Bakhtiari province. 324 swap poultry samples of different regions in Chaharmahal Va Bakhtiari province were collected and transferred to laboratory for PCR testing. Total DNA was extracted and then, *16S rRNA* region was amplified by PCR using specific primers. The results showed one amplified fragments, about 366 bp for positive cases. From 324 samples collected in this study, a number of 96 (29.63%) were positive on the basis of PCR analysis. The prevalence of *M. gallisepticum* infection was 30.50, 38.55, 22.81 and 18.60% in Shahrekord, Borujen, Farsan and Lordegan townships, respectively. The results of this study demonstrated the wide spread of *M. gallisepticum* in poultry and confirmed that infection was highly prevalent in Chaharmahal Va Bakhtiari province located in southwest Iran. These findings suggested that prevention and control programs includes chemotherapy and vaccination for reduce the distribution of *M. gallisepticum* infection is seems to be necessary in Iran.

Abbreviations: *M. gallisepticum, Mycoplasma gallisepticum*; PCR, Polymerase chain reaction

Key words: *Mycoplasma gallisepticum* · Poultry · PCR · Iran

INTRODUCTION

Mycoplasmas are widespread in nature as parasites of humans, mammals, reptiles, fish, arthropods and plants [1]. Mycoplasmas differ from other bacteria in their very small size and total absence of a cell wall; these characteristics account for their 'fried egg' type of colonial morphology, complete resistance to antibiotics that affect cell wall synthesis and their complex nutritional requirements. They tend to be host-specific, so that avian mycoplasmas are not generally known to infect mammalian or other species, but some non-avian mycoplasmas, such as *Mycoplasma bovis* [2], can cross the species barrier and have been found in avian species. The avian mycoplasma species that is pathogenic to commercial poultry, namely *Mycoplasma gallisepticum* (*M. gallisepticum*) and *Mycoplasma synoviae* (*M. synoviae*) in chickens and turkeys and *Mycoplasma meleagridis* (*M. meleagridis*) and *Mycoplasma iowae* (*M. iowae* in turkeys, have been associated with significant economic losses [3]. *M. iowae* has more antigenic diversity, can survive longer in the environment and is relatively more resistant to many antimicrobials than the other avian mycoplasmas [4]. The diagnosis of avian pathogenic mycoplasmas has typically been carried out by culture, serology and molecular-based assays such as polymerase chain reaction (PCR) and DNA probe. These tests each have advantages and disadvantages. Traditional culture isolation of mycoplasmas is time-consuming and complicated, requiring two to three weeks to complete because of the slow-growing nature of some mycoplasmas such as *M. iowae*. Birds infected with *M. iowae* do not consistently produce a humoral response that can be detected by the conventional serological tests that are used with the other avian mycoplasma pathogens [5]. 16S ribosomal RNA gene is a component of the 30S subunit of prokaryotic ribosomes and it is 1,542 nucleotides in length and widely use to

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molecular detection of microorganism such as mycoplasmas [6]. The avian pathogen *M. gallisepticum* induces severe chronic respiratory disease in chickens as well as sinusitis in turkeys [7, 8]. These diseases are globally prevalent and economically damaging to the Iranian poultry industry due to their effects on feed efficiency [9]. Although *M. gallisepticum* infections mainly affect the respiratory tract, they may occasionally cause keratoconjunctivitis [10], salpingitis [11], arthritis [12] and fatal encephalopathy [13]. The avian pathogen can be transmitted from infected birds via aerosol or via the egg [14]. The aim of this research was to PCR based determine the prevalence of *M. galliseopticum* among the poultry using *16S rRNA* gene in southwest Iran.

MATERIALS AND METHODS

Samples Collection and DNA Extraction: A total of 324 swap samples were collected from four townships of Chaharmahal Va Bakhtiari province (Shahrekord, Borujen, Farsan and Lordegan). In these poultry samples 141, 83, 57 and 43 specimens were obtained from Shahrekord, Borujen, Farsan and Lordegan townships, respectively.

Genomic DNA was extracted using DNA isolation kit (Gentra Inc., Minneapolis, MN) according to manufacturer's instructions. The total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell [15]. The extracted DNA of each sample was kept frozen at -20°C until used.

Gene Amplification: The PCR procedure was performed using a pair of oligonucleotide primers: M.Syn-F: 5'-GCGATGACGTGTAGTTATGCTG-3' and M.Syn-R: 5'-CCAATGCATACAATCGTTAAGC-3' chosen in the highly conserved regions of the published 16S rRNA sequences. Primers were designed according to the published sequence for *16S ribosomal RNA* gene of *M. gallisepticum* (accession number: M22441).

PCR amplification was carried out in 0.5ml tubes in a final reaction volume of 25 µl. The PCR mixture consisted of 100 ng of template DNA, 2 mM $MgCl_2$, 1 µl of each primers, 2.5 µl of 10X PCR buffer, 200 mM dNTPs and 1 U *Taq* DNA polymerase (Fermentas, Germany). The following conditions were applied: initial denaturation at 94°C for 5 min, 30 cycles, denaturation at 94°C for 1 min, annealing at 61°C for 1min, elongation at 72°C for 1 min, final elongation 72°C for 5 min. The samples were amplified in a Gradient Palm Cycler (Corbett Research, Australia). *M. gallisepticum* strain ATCC 19610 was used as positive control.

Analysis of PCR Products: The amplification products were analyzed in 1% agarose gel electrophoresis. The electrode buffer was TBE (Tris-base 10.8 g 89 mM, Boric acid 5.5 g 2 mM, EDTA (pH 8.0) 4 ml of 0.5 M EDTA (pH 8.0), combine all components in sufficient H_2O and stir to dissolve). Gels were stained with ethidium bromide. Aliquots of 10 µl of PCR products were applied to the gel. Constant voltage of 80 V for 30 min was used for products separation. After electrophoresis, images were obtained in UVI doc gel documentation systems (UK).

Statistical Analysis: The numbers of poultry presenting airsacculitis and the prevalence of re-isolation of *M. gallisepticum* from the swap were analyzed by the chi-square test using the SPSS 17 (SPSS Inc. Chicago, IL, USA) software. The probability level for significance was $P \leq 0.05$.

RESULTS

The quality of the extracted DNA from samples was examined by electrophoretic analysis through a 2% agarose gel. Four townships poultry have been investigated in Chaharmahal Va Bakhtiari province of Iran for the presence of *M. gallisepticum* infection (Shahrekord, Borujen, Farsan and Lordegan). The *16S rRNA* gene of *M. gallisepticum* was successfully amplified with the M.Syn-F and M.Syn-R primers. Agarose gel electrophoresis of the PCR amplified products is shown in Figure 1.

From 324 mycoplasma samples which assayed by PCR in this research, only 96 samples (29.63%) were positive (366 bp fragment). In Shahrekord, 43 poultry were found positive out of 141 poultry giving an apparent prevalence rate of 30.50%. In Borujen, 32 poultry out of 83 poultry was found to have *M. gallisepticum* infection. The apparent prevalence rate of *M. gallisepticum* was 22.81% in Farsan (13 out of 57) and only 8 poultry was found out of 43 poultry in Lordegan township (18.60%). These results were shown in Table 1. These finding showed that, there were significant differences among the flocks in relation to townships distribution using chi-square test ($P \leq 0.05$).

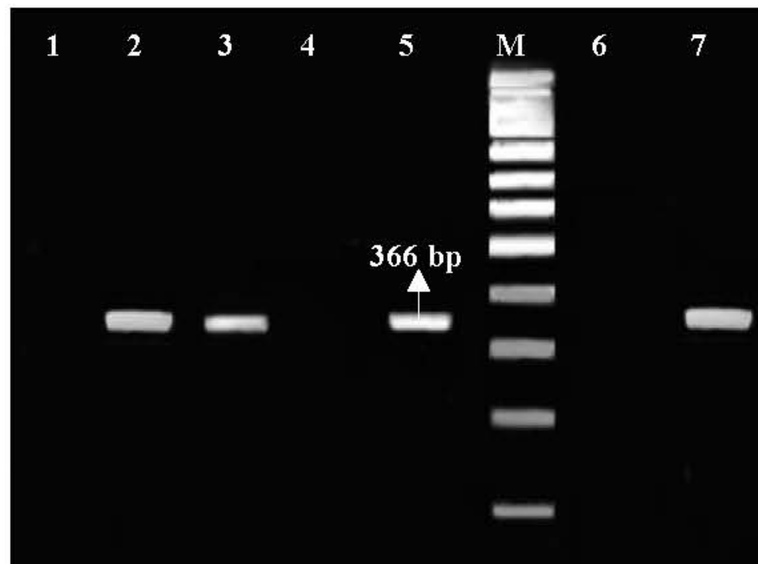

Fig. 1: Agarose gel stained with ethidium bromide, with PCR products of *M. gallisepticum* isolates. Lines 1 and 4 are *M. gallisepticum* negative samples, lines 2, 3 and 5 are *M. gallisepticum* positive samples, line M is 100 bp DNA ladder, line 6 is negative control and line 7 is positive control

Table 1: Prevalence of *M. gallisepticum* at poultry in Chaharmahal Va Bakhtiari province located in southwest Iran

Township	Number of samples	*M. gallisepticum* -negative, number (%)	*M. gallisepticum* -positive, number (%)
Shahrekord	141	98 (69.50%)	43 (30.50%)
Borujen	83	51 (61.45%)	32 (38.55%)
Farsan	57	44 (77.19%)	13 (22.81%)
Lordegan	43	35 (81.40%)	8 (18.60%)
Total	324	228 (70.37%)	96 (29.63%)

DISCUSSION

The wide occurrence of mycoplasmas has frequently led researchers with little or no expertise in mycoplasmology to suggest that structures resembling mycoplasmas in tissues of oysters, bryozoans and Giardia are mycoplasmas [1]. Yamamoto and Adler in 1958 described five serotypes, Kleckner in 1960 described eight serotypes, Yoder and Hofstad in 1964 characterized twelve serotypes and Dierks *et al.*, in 1967 described nineteen serotypes of Mycoplasma [16-19].

Different species of Mycoplasmas are *M. gallisepticum*, *M. iowae*, *M. synoviae*, *M. meleagridis*, *M. gallinarum*, *M. gallinaceum*, *M. cloacale*, *M. iners*, *M. gallopavonis* and *M. glycophilum* [20]. *M. gallisepticum* is responsible for chronic respiratory disease in chickens and infectious sinusitis in turkeys. Infections in commercial poultry flocks result in significant losses due to reduced egg production, poor feed conversion and carcass condemnation at processing [21]. *M. gallisepticum* is spread only short distances by the air-borne route. Where excellent biosecurity is practiced, there have been many instances where infection has not spread to adjacent houses within a complex.

M. gallisepticum infection is a chronic respiratory disease (CRD) in avian species [22]. Chickens, turkeys, quails, parrots, pheasants, pigeons and peacocks are the natural hosts of *M. gallisepticum* infection. The mortality rate is low unless a secondary microorganism infection is present. Infection with this bacterium causes sneezing, conjunctivitis, airsacculitis and decreased egg production in affected birds. The *M. gallisepticum* organisms in infected birds can be transmitted to the other birds via direct contact; that is, horizontal transmission. *M. gallisepticum* diagnosis can be carried out by various techniques using necropsy to observe gross and microscopic lesions, serology to determine the immune response, including SPA and ELISA tests and *M. gallisepticum* detection to find either the organisms or their DNA, using culture and isolation and PCR procedures [23].

Polymerase chain reaction (PCR) technique has increased the sensitivity of organism detection based on specific sequences of nucleotides [24]. In current study *M. gallisepticum* infection in Chaharmahal Va Bakhtiari province (southwest Iran) was found in 96 out of 324 (29.63%) samples. The prevalence of this pathogen was 30.50% in Shahrekord, 38.55% in Borujen, 22.81% in Farsan and 18.60% in Lordegan Townships. Mycoplasma pathogens cause respiratory and locomotory illness in poultry and other avian species. They are responsible not only for clinical diseases but also for decreased weight gain, lowered feed conversion efficiency, reduced hatchability and downgrading at slaughter [3].

Many studies were performed about *M. gallisepticum* in poultry and described its correlation with respiratory, locomotory illness and economic loses of this pathogen. Kempf et al. in a study on *M. gallisepticum* in 1993, showed that culture was positive for 49/73 swabs while PCR detected 70/72 positive samples [20]. Their used molecular technique for detection of *M. gallisepticum* infection and it was same to the method of present study and confirmed the results of our study in poultry samples. *M. gallisepticum* has been isolated from naturally occurring infections in chickens, turkeys, pheasants, chukar partridge, peafowl [25], bobwhite quail and Japanese quail [26]. *M. gallisepticum* has also been isolated from duck [27, 28]; from geese [27, 29]; from house finches [30], from a golden pheasant in Australia [31]; and from a yellow-naped Amazon parrot [32].

M. gallisepticum infection has been produced experimentally in captive-reared wild turkeys [33]. There are reports of *M. gallisepticum* detection in sera and isolations from wild turkeys [8, 34, 35] and various other free-flying birds including sparrows [36] but the significance of the presence of *M. gallisepticum* in these species has not been clearly established. Ghaleh Golab *et al.* in 2005 showed that PCR and RFLP are rapid and useful for diagnosis of both cultured as well as field samples of suspected flocks to have infection with *M. gallisepticum* [37]. In another study in Turkey (Ankara), Ongor *et al.* reported that mycoplasma growth was observed from 1.4% (9/624) of the samples, which were confirmed to belong to the *Mycoplasma* genus by genus-specific PCR. *M. iowae* and *M. meleagridis* were identified by the species-specific PCR from eight and one of the samples, respectively [38]. Feberwee *et al.* showed carried out a comparative study between several diagnostic methods (culture, PCR, SPA, HI and ELISA) for *M. gallisepticum* and *M. synoviae* and demonstrated a high number of false positive results in ELISA and SPA. From 712 sera positive in SPA, only 4.21% were positive in HI [39]. The results of their study confirmed the findings of current research.

In conclusion these findings suggested that *M. gallisepticum* was the likely cause of this outbreak of conjunctivitis in poultry. Also this infection can be transmitted naturally to an additional host species. The results of present study demonstrate a high spread of *M. gallisepticum* infection among poultry in Chaharmahal Va Bakhtiari province in southwest Iran. The control of this pathogen is useful for prevention and reduction of the incidence of Mycoplasmosis and reduce the economic loses in this region.

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