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Application of Molecular and Serological Methods for Rapid Detection of *Mycoplasma gallisepticum* Infection (*Avian mycoplasmosis*)

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ABSTRACT
Mycoplasma infection is a major problem in veterinary medicine and in poultry production. The pathogen has many strains, so that diagnosis of the disease using culture method is not effective. The objective of this work was to evaluate the prevalence of *Mycoplasma gallisepticum* (MG) in Kuwait poultry farms using serology and molecular methods in comparison to the culture under specific conditions. A total of 50 swab samples from choanal cleft and tracheal samples and blood samples were obtained from three different local farms, the blood samples were processed for an Enzyme Linked Immunosorbent Assay (ELISA) detection and the swab samples for Polymerase Chain Reaction (PCR) and culture methods detection. A PCR diagnostic kit (VenoMGs) and ELISA diagnostic kit (ProFLOK), were used in comparison to the traditional culture method, to study the spread of this disease in samples from broiler and layer flocks. Fifty chicken samples were tested for mycoplasmosis, samples tested with ELISA gave 24 positive (48%) and 29 were positive by PCR (58%) and only seven (14%) were positive with culture methods. Swab samples obtained from the choanal cleft gave more positive (60%) with PCR than tracheal samples (56.6%). The culture gave 20 and 5% positive, respectively for tracheal and choanal samples. The methods reported here are of high sensitivity and specificity for Mycoplasma. Both the PCR and ELISA methods are superior to culture method for detection of avian mycoplasmosis. This study showed that MG infection is prevalent in commercial broiler and layer chickens in Kuwait poultry farms. The use of these methods for surveillance of the disease will establish data concerning the predominant Mycoplasmosis diseases in Kuwait if done on a large scale.

Key words: Kuwait, PCR, ELISA, *Mycoplasma gallisepticum*, Mycoplasmosis, Avian

INTRODUCTION

Chronic colonization of the major airways with Mycoplasma will lead to devastating exacerbations of pulmonary infections, this is the main cause of morbidity and mortality in chickens with Chronic Respiratory Disease (CRD), (Levisohn and Kleven, 2000; Nascimento *et al*., 2005). *Mycoplasma gallisepticum* (MG) represents a pathogenic species within the genus *Mycoplasma* that belongs to the family *Mycoplasmataceae* and order *Mycoplasmatales* of the class *Mollicutes* (Ley, 2008).

Mycoplasma gallisepticum produce small, smooth, circular translucent colonies on agar plates with Frey's medium, or a modification of Frey's medium (Kleven, 2008), with a dense, raised central area (Ley, 2008). *Mycoplasma* pathogens cause respiratory and locomotory illness in chickens

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 (Bradbury and Kleven, 2008).

The MG economically impacts the poultry industry through increased mortality decreased egg production (Carpenter *et al*., 1981; Parker *et al*., 2002) and reduced feed efficiency (Ley, 2008). Increased medication costs are additional factors that make this disease one of the costliest disease problems for poultry industry (Mohammed *et al*., 1987).

Mycoplasma gallisepticum infection has become an emerging problem for commercial chicken flocks, particularly those reared in a constant production site. However, there is some evidence that MG is also present in small backyard poultry flocks (Herrero *et al*., 2009). Serum Plate Agglutination (SPA) test, Hemagglutination Inhibition (HI) test and Enzyme-Linked Immunosorbent Assay (ELISA) have been used to detect MG and MS (Parker *et al*., 2002; Mohammed *et al*., 1986; Kang *et al*., 2002). The infection appears to be worldwide in distribution (Ley, 2008).

Laboratory methods are essential to diagnose MG, since clinical signs or pathological lesions cannot diagnose MG infection. There are three approaches to diagnose MG which are: isolation and identification of the organism, detection of its DNA and detection of specific antibodies (Bradbury and Kleven, 2008).

Polymerase Chain Reaction (PCR) technique has increased the sensitivity of organism detection based on specific sequences of nucleotides (Nascimento *et al*., 1993; Slavik *et al*., 1993). Serologic procedures are useful for flock monitoring in MG control programs and to aid in diagnosis when infection is suspected (Ley, 2008). Enzyme Linked-immunosorbent Assays (ELISAs) have been used to detect MG antibodies in respiratory tract washings (Avakian and Ley, 1993; Yagihashi and Tajima, 1986) and egg yolk samples (Mohammed *et al*., 1986).

The aim of this study was to compare several methods for *M. gallisepticum* diagnosis. The results of this study would be useful for fast and suitable *M. gallisepticum* diagnosis in poultry farms in Kuwait and it can help also in the prevention and control of *M. gallisepticum* infection.

MATERIALS AND METHODS

Field specimens: Endo-tracheal, choanal cleft swabs and serum were collected from asymptomatic chicken and chickens suffering from respiratory symptoms.

Culture methods: For culture and isolation the procedure followed was as described by Kleven (2008). Cotton swabs were transferred into 4 ml in Pleuro Pneumonia like Organisms (PPLO) broth and agar supplemented with *Mycoplasma* supplements (DIECO) and glucose (0.1%) plus phenol red (0.002%) to serve as an indicator for growth. The cultures were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. The change in pH resulted in color change due to metabolic activity of the mollicutes (cells) as they ferment glucose if growth proceeds.. One week after incubation, the unchanged cultured broth was passed to a new PPLO broth, followed by one more passage if the color was unchanged. For the changed cultured broth, the broth was streaked onto PPLO agar containing 15% swine serum, then incubated at 37°C with humidity, The cultures were examined microscopically at 2, 5, 10, 15, 25 and 30 days of incubation for the presence of typical colonies using inverted microscope. The Mycoplasma colonies were confirmed as MG by a direct immunofluorescence test (Kleven, 2008). The standard *M. gallisepticum* strain ATCC 15302 (American type culture collection, Atlanta, USA) was grown as a control.

Molecular diagnosis: The Venor®MGS *Mycoplasma gallisepticum* diagnostic kit (Minerva Biolabs GmbH, Berlin, Germany) has been used. The Venor®MGS screening assay is a multiplex PCR system for detection and specification of mycoplasma species associated with poultry diseases. The kit includes a Positive control *M. gallisepticum* (DNA-fragments, prepared by PCR, non-infectious, lyophilized Internal control (Lyophilized plasmid DNA, including Mycoplasma-specific primer sequences and an internal sequence of the HTLV-I tax gen with a size of approx. 210 bp, non-infectious).

Test principle: Venor®MGS-*Mycoplasma gallisepticum* diagnostic kit utilizes the Polymerase Chain Reaction (PCR) which was established as the method of choice for highest sensitivity in the detection of Mycoplasma infections in poultry. Venor®MGS diagnostic requires as little as 1 to 5 fg of Mycoplasma DNA, corresponding to 2-5 Mycoplasma per sample volume. The supplied primer set is specifically for a highly conserved membrane protein coding segment of the genome of each pathogen. The amplified PCR product expected for *Mycoplasma gallisepticum* was 281 bp long.

Preparation of sample material: Swabs from the trachea and the choanal cleft of a chicken were placed into a tube containing 0.5 mL Phosphate Buffer Saline (PBS), washed gently, the excess liquid was squeezed from the swab by rolling the swab against the wall of the tubes, heated for 15 min at 95°C, the samples were stored in ice and tested within 24 h. For 50 μL PCR reaction the mixture was as follows: Ultrapure H_2O (27.8 μL), 5011OX reaction buffer, 0.2 μL *Taq* polymerase enzyme (5 U mL^{-1}), $mgcl_2$ (50 mM), 5.0 μL primer/nucleotide mix, 2.0 μL internal control. The experiments included positive and negative controls, for negative control, sterile water was added instead of nucleic acid. *M. gallisepticum* strain ATCC 15302 ATCC 19610 were included as a positive control.

Amplification was carried out in a thermal cycler (GeneAmp PCR system 9700, Perkin, Elmer, Cetus, USA). PCR running program was performed as follows: One cycle 94°C for 2 min, 55°C for 2 min, 72°C for 2 min, 34 cycles 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min, 1 cycle 72°C for 4 min, cool down to 4°C.

The amplified products were separated by 1.5% standard agarose gel with 5 mm-comb was used, 5 µL of each PCR reaction was loaded, mixed with bromophenol blue loading buffer per lane; the electrophoresis were stopped after 2 cm run distance (the run was for 20 min at 100V). The PCR products were resolved by agarose electrophoresis and photographed under UV light using standard procedures (Qasem *et al*., 2002).

Immunological diagnosis: Serology Determination of *Mycoplasma*-specific antibodies (IgG, IgA, IgM) in the sera was performed by Enzyme Linked Immunosorbent Assay (ELISA) using a commercial kit (ProFlok®MG ELISA, SynBiotics Co, USA) according to the manufacturer's recommendations and directions. At least 50 sera per flock were randomly collected at standard time intervals. Serum sample storage (4°C for up to four days or -20°C for longer periods) was used to provide reliable test results. The serum samples were diluted using Dilution Buffer in a clean, uncoated 96 well microtiter plate. Briefly, diluted sera were added onto an MG antigen-coated plate, incubated, washed and peroxidase labeled, anti-chicken antibody (conjugated antibody) was added. After incubation, the plate was again washed before adding a substrate and adding the stock solution. The plate was read in an ELISA reader (Labsystems Multiskan MS Type 352, Finland). The optical density of the negative, positive controls and samples were calculated and interpreted according to the manufacturers' recommendations. For the interpretation of ProFlok®MG ELISA, titer levels 0-148, 149-743 and equal or higher than 744 were negative, suspicious, or positive reactors, respectively. For the interpretation of ProFlok®MG ELISA, titer levels 0-1075 and levels equal or higher than 1076 were negative, or positive, respectively.

Ethical statements: All birds were used under the auspices of a protocol approved by Public Authority for Agriculture and Fish Resources (PAAF), Kuwait.

RESULTS

Fifty commercial flocks were surveyed for avian mycoplasmosis, they comprised 24 chicken broiler flocks and 26 layer chicken flocks.

Various methodologies for Mycoplasma detection were compared in this study. When only Mycoplasma cultures (reference strains) were tested using, the type culture method and PCR methods, the organism with no discrepancies was detected (Fig. 1 and 2). Out of 50 different chicken samples, 6 gave positive results with all of the methods used and 15 gave positive results with 2 of the three test methods used (Table 1). Of the fifty chicken samples tested for Mycoplasma using the culture method and detection kits, culturing gave seven positive samples (14%) (Table 2), PCR gave 29 positive samples (58%) (Table 2), serum test with ELISA gave 24 positive samples (48%).

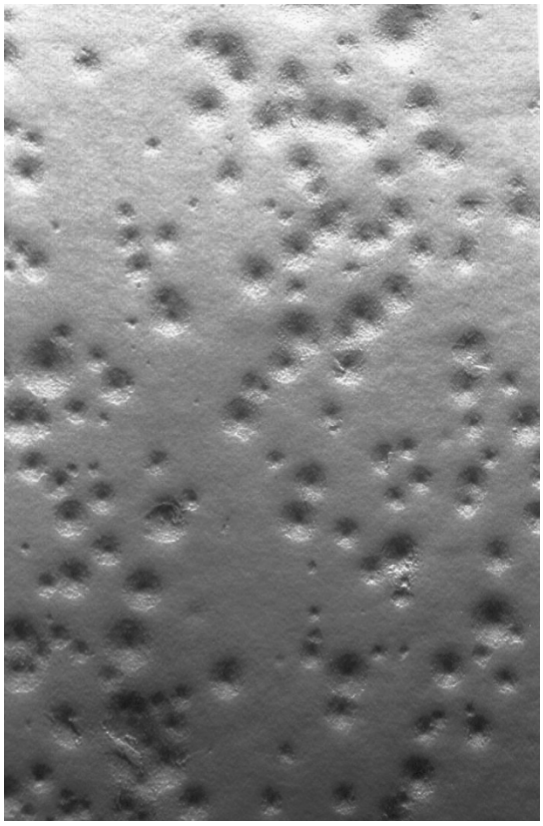

Fig. 1: Compound light microscope image of *Mycoplasma gallisepticum* colonies on PPLO Agar Medium visualization under stereomicroscope (40×) showing typical "Fried egg"morphology

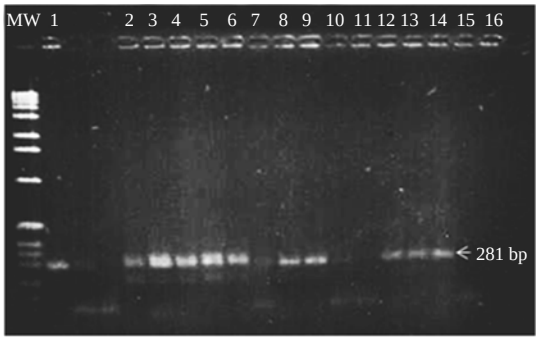

Fig. 2: PCR amplification of swab samples of chicken. Lane MW: DNA, LCB ladder, Lane 1: Positive control, Lane 2 and 3: Negative control Lane 4-16: Swab sample

PCR results were as follows: *M. gallisepticum*, positive sample, highly infected gave strong band at 281 bp, *M. gallisepticum* positive sample with weak infection gave band at 281 bp and at 210 bp, negative sample gave band at 210 bp (Fig. 2).

Table 1: Results obtained by method of Diagnosis of Mycoplasma in chicken

			Diagnostic methods		
Sample type	Chicken type	Chicken source	Culture	PCR	ELISA
M. gallisepticum	Ref	ATCC15302	+	+	+
M. gallisepticum	Ref	ATCC19610	+	+	+
TS	Broiler	Naife	-	+	+
CCS	Layer	Naife	-	+	+
CCS	Layer	Naife	+	+	+
TS	Layer	Naife	+	+	+
CCS	Layer	KUPCO	-	+	+
TS	Layer	KUPCO	+	+	+
TS	Broiler	KUPCO	-	-	-
CCS	Broiler	KUPCO	-	+	-
TS	Broiler	KUPCO	-	+	-
TS	Layer	KUPCO	-	-	-
TS	Broiler	Naife	-	-	-
TS	Layer	Naife	-	+	+
CCS	Layer	Naife	-	+	+
TS	Layer	Abo Ali	+	+	+
TS	Broiler	Abo Ali	-	-	-
CCS	Broiler	Abo Ali	-	-	-
TS	Broiler	Naife	-	-	-
TS	Broiler	Naife	+	+	+
CCS	Broiler	KUPCO	-	+	-
CCS	Broiler	KUPCO	-	+	-
CCs	Broiler	KUPCO	-	+	+
TS	Broiler	KUPCO	-	+	+
CCS	Layer	KUPCO	-	-	-
TS	Layer	Abo Ali	-	+	+
CCS	Layer	Abo Ali	-	+	+
TS	Layer	Abo Ali	-	-	-
TS	Layer	Abo Ali	-	-	-
TS	Broiler	Naife	+	+	+
CCS	Broiler	Naife	-	+	+
TS	Broiler	Naife	-	+	+
CCS	Broiler	Naife	-	-	-
CCS	Layer	Naife	-	-	-
TS	Layer	Naife	-	-	-
CCS	Layer	Naife	-	-	-
TS	Broiler	Naife	-	-	-
TS	Broiler	Naife	-	Na	-
TS	Broiler	Naife	-	Na	-
TS	Layer	Naife	-	Na	-
CCS	Layer	Naife	-	Na	-
CCS	Layer	Naife	-	Na	-
TS	Broiler	Abo Ali	-	+	+
CCS	Broiler	Abo Ali	-	-	-
TS	Layer	Abo Ali	-	-	-
TS	Layer	Abo Ali	-	+	+
TS	Broiler	Abo Ali	-	+	+
TS	Layer	KUPCO	+	+	+
CCS	Layer	KUPCO	-	+	+
CCS	Layer	KUPCO	-	+	+
TS	Layer	KUPCO	-	+	+
TS	Broiler	KUPCO	-	+	+

TS: Tracheal swab, CCS: Choanal cleft swab

Table 2: Comparison of the three detection methods results between Broiler and Layer Flocks

		Diagnostic method used							
		Culture		PCR		ELISA		Total	
Chicken type positive	Total tested	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Broiler	24	2	8	14	58	10	42	14	58
Layer	26	5	19	15	59	14	54	16	62
Total	50	7	14	29	58	24	48	30	60

Out of the 24 broiler flock tested only two (8%) were positive with culture, 14 (59%) with PCR and 10 (42%) with ELISA. Among the layer flocks 16 gave positive out of 26 samples, five (19%) with culture, 15 (58%) with PCR and 14 (54%) with ELISA. When the source of the sample swab was compared with the PCR test, 17 of total 30 tracheal swabs (56.6%) were positive out of total 30 samples and 12 of choanal cleft samples were positive (60%) out of 20 total

Table 3: Comparison between the three testing methods in relation to the source of the sample

Testing method	Total positive	Total tested	Percentile (%)
PCR			
Tracheal Swab	17	30	56.6
Choanal Cleft swab	12	20	60.0
Culture			
Tracheal swab	6	30	20.0
Choanal Cleft Swab	1	20	5.0

samples (Table 3). For culture technique, only one sample was with positive growth on PPLO agar with inoculation from choanal cleft and six from tracheal swaps (Table 3).

DISCUSSION

Mycoplasma gallisepticum (MG) seroprevalence in chickens has been reported to be high in many countries with no control strategy or in countries before the implementation of a control strategy. For example, MG seroprevalence was 73% in layers in Southern California in 1984 (Mohammed *et al*., 1986) in the Middle East region, significant seroprevalence of the disease was evident in Egyptian chickens (Saif-Edin, 1997) and it was 73.5% in northern Jordan (Gharaibeh and Al Roussan, 2008).

Diagnosis of MG can be carried out by different techniques using autopsy to observe gross and microscopic lesions, serology can be used to determine the immune response, comprising SPA and ELISA tests and the detection of the organism or their DNA, using culture isolation and PCR procedures (Ley, 2008).

Isolation and/or identification of the organism is the gold standard for *Mycoplasma gallisepticum* infection diagnosis in chickens but this procedure is time consuming, laborious and some field isolates taken longer and require a few serial passages (Ley, 2008). In this study, MG infection in flocks was 42% by ELISA and 58% by PCR and 8% by isolation in broiler chicken and 54, 58 and 19% of layer chicken respectively. The low detection using culture method could be due to the over-growth of other bacteria on the agar plates which suppresses the appearance of typical colonies of Mycoplasma, could have caused the discrepancy (Parker *et al*., 2002). In comparison to other types of chickens, layer flocks had higher infection with *Mycoplasma gallisepticum*. This is possibly due to longer life span of a layer than broiler flocks increasing the chance for *Mycoplasma gallisepticum* field exposure.

The sero-prevalence and molecular study revealed the occurrence of MG infection in commercial chickens in Kuwait. This is comparable to what is reported in some Arab countries (Gharaibeh and Al Roussan, 2008; Gharaibeh *et al*., 2011; Saif-Edin, 1997) However, the losses caused by MG were not estimated in this study. Several researchers demonstrated the average egg production loss due to naturally exposed MG infection, was 15.7 eggs/hen as compared with MG-free hens (Carpenter *et al*., 1981). Other researchers presented that MG-infected flocks produced 5-12 less eggs per hen compared with uninfected flocks (Mohammed *et al*., 1987). From this information, it can be assumed that how MG can impact on commercial poultry production. Further study is necessary to find out the impact of MG on commercial poultry production system in Kuwait.

The precondition for successful production of commercial poultry, free from MG includes establishment of a monitoring program for MG and separation of MG infected and free flocks at the top of the production pyramid and complete separation of eggs from these flocks in the hatcheries. In addition, the impact of biosecurity has to be underlined, for this reason a very sensitive and selective method of diagnosis is important (Ley, 2008).

Flock free of MG license and certification in a basic line flock of birds is thus defined for the control of the pathogen. In many cases this might not be as simple, since usually no clinical signs are apparent, laboratory test must be highly reliable. They must be sensitive enough so as not to result in false negative results but also specific enough so as not to result in false-positive (Kleven, 2008). ELISA test kit ProFlok is presently used for flock monitoring and serodiagnosis (Ley, 2008). In addition, using PCR for detection of MG has been proven as a rapid, sensitive and specific diagnosis (Nascimento *et al*., 2005; Salisch *et al*., 1998; Slavik *et al*., 1993).

Previous investigations of the prevalence of MG in commercial layer chickens showed that MG infection represents a major problem for chickens reared in commercial poultry farms and Government farms (Pradhan *et al*., 2000; Saleque *et al*., 2003). This study showed that MG infections are prevalent in commercial broiler and layer chicken in Kuwait and thus there is a need for a better understanding of the epidemiology of Mycoplasma G infection in broiler breeders in Kuwait. Several features of the MG infection remain distorted, because there are no clinical signs of the disease and thus application of a dependable method is needed (Hossain *et al*., 2007).

As expected, a higher estimate of the prevalence of exposure to MG in the sample population from the serological survey was obtained than from the clinical observation data, prior studies have documented that the ELISA and PCR assays are useful in confirming clinical findings (Hartup and Kollias, 1999; Hartup *et al*., 2000; Luttrell *et al*., 1996). Rapid, inexpensive serological assays are extremely useful in large-scale epidemiological studies and provide a potential means of identifying birds that are infected but asymptomatic as well as recovering birds. Although, the ELISA assay is rapid and relatively inexpensive. Nonspecific reactions have been reported in poultry and there are no data on the occurrence of false positive reactions in commercial chicken (Ley, 2008; Luttrell *et al*., 1996). The results demonstrate that confirmation of positive serological results by PCR permitted the zero detection of asymptomatic infected birds and provides a more complete estimate of the prevalence of MG infection.

A range of factors, such as timing and randomness of serum sample collection procedure, possible M strain variations that may exhibit atypical biological and/or antigenic properties, could consequently result in an M-infected chicken flock yielding M negative ELISA results (McLaren *et al*., 1996).

It has been suggested earlier that MG surveillance should not be solely based on SPA but it should be supported by PCR and attempts to isolate the agent (Ewing *et al*., 1998).

The studies suggested that early interventions may help decrease the associated morbidity and mortality of chickens with CRD. It is therefore important that primary diagnostic bacteriology laboratories have the ability to detect transient and early MG colonization as early as possible (Ewing *et al*., 1998).

CONCLUSION

To date, this is the first report to describe the detection of MG in Kuwait commercial farms using three methods of detection (ELISA, PCR and culture). These methods will be very helpful in establishing a baseline data on the predominance of the disease in poultry industry in Kuwait, especially in a wide range program surveillance. Such information is a prerequisite for future regional and international collaboration to identify the source of etiological agents, leading to the control of their spread among the farms in the country.

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