ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF POULTRY SCIENCE

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International Journal of Poultry Science 8 (2): 108-111, 2009
ISSN 1682-8356
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Detection of *Mycoplasma gallinarum* by Real-Time PCR

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Abstract: *Mycoplasma gallinarum* colonizes poultry as well as mammals, but is considered to have a commensal relationship with its hosts. Though unable to cause poultry disease by its self, reports have been published suggesting a synergism during mixed infections between *M. gallinarum* and respiratory viruses or their vaccine strains that can precipitate airsacculitis. Currently, little research is being done on *M. gallinarum* and little is known about its carrier rate in chickens and other poultry. Two primer sets were tested for their ability to detect *M. gallinarum* using real-time PCR. One set published by Lauerman amplifies a fragment from the *M. gallinarum* 16S ribosomal DNA sequence. The other set (101-2) was developed in this laboratory and amplifies a short segment of DNA that appears to be unique to some strains of *M. gallinarum*. The Lauerman primer set is specific for *M. gallinarum* and has a detection limit of 100 genomes. The 101-2 primer set is specific for some strains of *M. gallinarum*, although, it is 100-fold less sensitive than the Lauerman primer set. The 101-2 primer set appears to be unsuited for *M. gallinarum* detection, but it does provide a method of differentiating *M. gallinarum* strains by PCR. These primer sets provide a means to rapidly determine the *M. gallinarum* carrier status of flocks by real-time PCR and will help in identifying *M. gallinarum* in mixed infections.

Key words: *Mycoplasma gallinarum*, PCR, detection

INTRODUCTION

Mycoplasma gallinarum is considered to be a non-pathogenic commensal for a broad range of hosts. These hosts can include swine, sheep and cattle; although, the host range for *M. gallinarum* is commonly thought to be avian species (Robinson and Dinter, 1968; Robinson *et al*., 1968; Bencina *et al*., 1987; Singh and Uppal, 1987; Wang *et al*., 1990; Dovc *et al*., 1991). Compared to *Mycoplasma gallisepticum*, *M. gallinarum* produces little to no pathology (Power and Jordan, 1976). In fact, infection with *M. gallinarum* has been linked to delayed onset of fatty liver hemorrhagic syndrome (Branton *et al*., 2003). Little research is performed on *M. gallinarum* because it is considered to be non-pathogenic.

Despite, its non-pathogenic nature, some researchers have suggested a pathogenic role for this bacterium. Research reports have been published linking *M. gallinarum* to respiratory diseases in poultry, where it appears to serve as a cofactor in combination with pathogenic respiratory viruses including Newcastle disease virus and infectious bronchitis virus (Kleven *et al*., 1978; Bradbury, 1984; Shah-Majid, 1996). The ability to serve as a cofactor for pathogenic respiratory viruses is not surprising. However, *M. gallinarum* can also induce airsacculitis in broilers when the broilers are treated with infectious bronchitis virus and Newcastle disease virus vaccines (Kleven *et al*., 1978). Similar results have been obtained using *M. gallisepticum* and *Mycoplasma synoviae* (Kleven *et al*., 1978). The current lack of information on *M. gallinarum* makes further study of its pathogenic potential warranted.

Current methods of detecting *M. gallinarum* include the presence and size of surface immunoglobulin receptors and PCR for 16S ribosomal DNA (Lauerman *et al*., 1993; Lauerman *et al*., 1995; Lauerman, 1998). The former method requires pure cultures and is time consuming. PCR with 16S rDNA specific primers provides a more rapid means of detection and can be used with mixed populations. Once *M. gallinarum* is detected, differentiation of strains is more difficult. The principle method of strain differentiation is based on restriction fragment length polymorphism (RFLP) analysis of PCR products generated using universal primers for the mycoplasma 16S/23S ribosomal DNA intergenic spacer region (Dovc *et al*., 1991).

In order to facilitate research of *M. gallinarum*, real-time PCR conditions for detecting *M. gallinarum* using the 16S rDNA primer set were developed. An alternative PCR primer set was also developed and while not useful for strain detection, does provide a means of differentiating some *M. gallinarum* strains by PCR.

MATERIALS AND METHODS

Bacterial strains: *Mycoplasma gallinarum* DNA was obtained from strains K3390, K285, K5466A and K5633, which were a generous gift from Dr. Stanley Kleven. *Mycoplasma gallisepticum* (MG) DNA was obtained from commercial vaccines FVAX-MG® (F-strain) (Fort Dodge Animal Health, Overland Park, KS), Mycoplasma Gallisepticum vaccine® (ts-11) (Merial Select, Gainesville, GA) and Mycovac-L® (strain 6/85) (Schering Plough Animal Health, Omaha, NE). Low passage MG

strain R-low was a generous gift from Dr. Steven Geary. *Mycoplasma gallinaceum* DNA was obtained from strain K3892F, a generous gift from Dr. Stanley Kleven. *Mycoplasma gallinarum* DNA used for cloning and sequencing was isolated from a strain previously obtained from Dr. Stanley Kleven (Wan *et al.*, 2004).

Real time PCR: Real time PCR was performed using an Applied Biosystems 7500 system (Applied Biosystems, Foster City, CA). SYBR® Green PCR master mix was obtained from Applied Biosystems. PCR for 16S rDNA was performed using *M. gallinarum* specific primers (Lauerman, 1998) and the following program [95°C 10 min, 94°C 15 sec, 55°C 20 sec, 72°C 60 sec (40 cycles)] (followed by melt-curve analysis. PCR using primers 101:GAATCTGGCTATGATAAATCTGCATACTAT and 102:CGCCTTGTCTAAATACACCTGTATAGTC was performed using the following program [95°C 20 sec, 65°C 20 sec, 72°C 75 sec (40 cycles)] followed by melt-curve analysis. Following real-time PCR, data were analyzed using the automated analysis function of the 7500 system software. Genome equivalents were calculated based on a genome size of 727 kbp (Herrmann, 1992) and were calculated to be 10 genomes per 7.46 fg of genomic DNA, which was used for standard curve generation.

Cloning and sequencing: *M. gallinarum* DNA was isolated from bacteria using a QIAamp DNA mini kit (Qiagen Inc., Valencia, CA). Genomic DNA was digested with PstI and cloned into the pZERO-2 plasmid (Invitrogen Corporation, Carlsbad, CA). Plasmid DNA from random clones was isolated using a QIAprep mini kit (Qiagen Inc.) and sequenced (Retrogen Inc., San Diego, CA). One of the DNA sequences obtained had minimal homology to any sequences in the GenBank database. PCR primers 101 and 102 were designed to amplify a 583 bp fragment specific to *M. gallinarum*. This sequence is deposited in GenBank as accession FJ404789.

RESULTS

To facilitate detection of *Mycoplasma gallinarum* with increased speed, a real-time PCR assay was developed using 16S rDNA specific primers developed by Lauerman (1998). Serial 10-fold dilutions of genomic DNA were analyzed to determine the sensitivity of the assay. Using conditions modified for the real-time PCR system, the sensitivity was found to be approximately 100 genome equivalents (Fig. 1 and Table 1) with a dissociation temperature of approximately 83°C (Table 1). Similar results were obtained over repeated experiments (data not shown). This primer set was shown to detect all four strains of *M. gallinarum* tested (Fig. 2). The Lauerman primer set was also shown to be specific for *M. gallinarum* as it was unable to detect common *M. gallisepticum* strains or *M. gallinaceum*, another avian mycoplasma of limited pathogenicity (Fig. 2).

Table 1: Average threshold cycle (Ct) values for quantitative detection of *M. gallinarum* using the 16S rDNA Lauerman primer set

Genome equivalents[a]	Ct value	Dissociation T_m
9750000	15.86	83.0
975000	20.13	83.4
97500	24.19	83.4
9750	27.94	83.7
975	31.75	83.7
97.5	35.79	83.7

[a]Genome equivalents were calculated from a published estimate of the *M. gallinarum* genome size

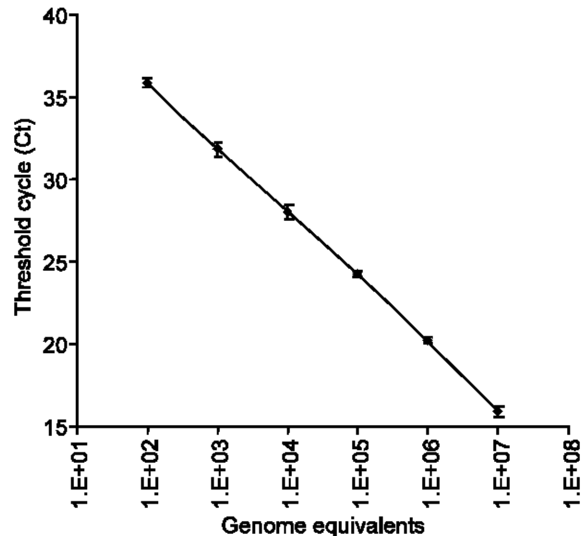

Fig. 1: Standard curve of 10-fold serial dilutions of *M. gallinarum* strain K5446A. PCR was performed using the 16S rDNA specific primers. Results are averaged from duplicate samples. Error bars represent the standard deviation in Ct values

A second primer set for detection of *M. gallinarum* was developed based on DNA sequence that contained minimal homology to other sequences in the GenBank database. The 28% GC content of this fragment is consistent with it being of mycoplasmal origin. However, unlike the 16S rDNA Lauerman primer set, detection of *M. gallinarum* using primers 101 and 102 was found to be specific for strain K5633, as no PCR product was obtained from the other 3 *M. gallinarum* strains or control DNAs tested (Fig. 3). It was also shown to detect the *M. gallinarum* strain from which the genome DNA was obtained (data not shown). Upon testing this primer set, it was found to be 100 fold less sensitive than the Lauerman primer set and to have a dissociation temperature of 76°C.

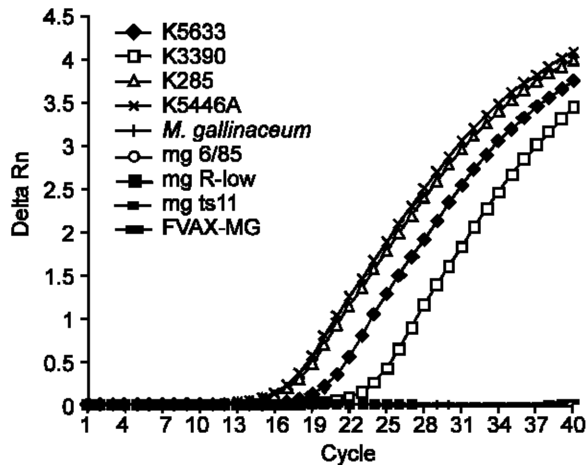

Fig. 2: Detection of *M. gallinarum* strains, control Mg (*Mycoplasma gallisepticum*) strains and *M. gallinaceum* using the 16S rDNA Lauerman primer set

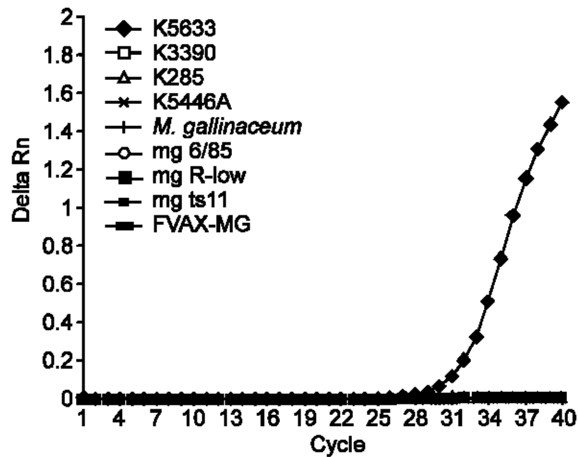

Fig. 3: Detection of *M. gallinarum* strains, control Mg (*Mycoplasma gallisepticum*) strains and *M. gallinaceum* using the 101-2 primer set

DISCUSSION

The primary goal of this work was to develop a real-time PCR assay for detection of *M. gallinarum* using the single established primer set as well as creating a new primer set. Real-time detection of *M. gallinarum* using the 16S rDNA Lauerman primer set was shown to be specific and effective for *M. gallinarum*. The results presented here show a detection limit of approximately 100 genome copies. Although, 10 genomic copies could not be detected based on the automated analysis parameters, a PCR product is produced at that dilution that appears to have a threshold Ct value of 39 and dissociation temperature of approximately 83°C. This suggests that 10 genome copies are just beyond the limits of detection. However, it is likely that 20-30 copies would be detected by the automated analysis parameters.

The 101-2 primer set proved to be of interest as, it permits *M. gallinarum* strain differentiation based on DNA sequence differences. The lack of a PCR product from other *M. gallinarum* strains tested suggest that genetic variation can be used as a tool to identify different *M. gallinarum* strains and may also provide important clues into differences in *M. gallinarum* pathogenic potential. These results provide a means for rapidly examining the *M. gallinarum* status of flocks. Differentiation of *M. gallinarum* strains using the 101-2 primer set is not likely to be very useful under field conditions, but is perhaps more applicable to research performed under laboratory conditions.

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