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Development and evaluation of a diagnostic PCR for *Mycoplasma synoviae* using primers located in the intergenic spacer region and the 23S rRNA gene

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Abstract

Mycoplasma synoviae (Ms) is an important pathogen of poultry, causing economic losses to this industry. Early and reliable diagnosis is a key to controlling the spread of this organism. In this study, a polymerase chain reaction with one primer based on the intergenic spacer region (ISR) was validated for detection of Ms. The ISR primer was paired with a general primer from within the 23S rRNA gene. The PCR primers were tested with the 22 other recognised avian *Mycoplasma* species to check the specificity and with 21 field isolates of Ms from various hosts and countries, and with several swab samples. The PCR appeared to be specific and sensitive. Four different sample preparation methods were compared for use in this PCR, and the amplification protocol was compared with three others, confirming the comparative sensitivity of the new PCR.

Keywords: *Mollicutes*; *Mycoplasma*; *Mycoplasma synoviae*; Diagnosis; Polymerase chain reaction

1. Introduction

Mycoplasma synoviae (Ms) is an economically important pathogen of poultry, causing respiratory tract infection and sometimes leg problems. Poultry breeding companies invest considerable resources into maintaining their flocks free from this mycoplasma.

Traditionally Ms has been diagnosed by serological screening or by culture followed by identification of the mycoplasma. Screening is relatively inexpensive but is not always sufficiently specific or sensitive while culture can be insensitive and time consuming (Kleven, 2003). Rapid and accurate identification of Ms is of great importance and molecular methods such as the polymerase chain reaction (PCR) have been developed to improve this. Earlier PCRs were based on the 16S rRNA gene (Lauerman et al., 1993; Garcia et al., 1996) and more recently, some have been based

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on haemagglutinin genes (Hong et al., 2004; Hammond et al., 2004; Ben Abdelmoumen Mardassi et al., 2005). In earlier work the intergenic spacer region (ISR) located between the 16S and 23S rRNA genes was sequenced for *M. gallisepticum* and *M. imitans* (Harasawa et al., 2004) and for the remaining avian mycoplasmas (Ramirez, A.S., Naylor, C.J., Pitcher, D., Bradbury, J.M., unpublished data, GenBank AJ780982–AJ781002,) and it was found that in this sequence there was greater inter-species variation than in the 16S RNA gene due to the fewer evolutionary constraints on the region (Barry et al., 1991). The ISR has been the target of PCRs to detect mycoplasmas in cell cultures (Harasawa et al., 1993), to detect *M. pulmonis* in clinical isolates (Takahashi-Omoe et al., 2004), *M. felis* directly in feline clinical samples (Chalker et al., 2004), and to identify phytoplasmas in infected plants (Smart et al., 1996).

We therefore decided to investigate the use of the ISR as a target for detection of Ms and we designed two forward primers, both based in the ISR and paired with a reverse primer based in the 23S gene. The aim of this study was to validate these tests by checking them with the type strains of 23 recognised avian *Mycoplasma* species and with a collection of Ms field isolates from different host species and geographical locations, and which were isolated over a number of different years.

2. Materials and methods

2.1. Organisms and culture conditions

The type strains of avian *Mycoplasma* species used were as follows: *M. anatis* 1340^T, *M. anseris* 1219^T, *M. buteonis* BbT2g ^{T}, *M. cloacale* 383^T, *M. columbinasale* 694^T, *M. columbinum* MMP-1^T, *M. columborale* MMP-4^T, *M. corogypsi* Bv1^T, *M. falconis* H/T1^T, *M. gallinaceum* DD^T, *M. gallinarum* PG16^T, *M. gallisepticum* PG31^T, *M. gallopavonis* WR1^T, *M. glycophilum* 486^T, *M. gypis* B1/T1^T, *M. imitans* 4229^T, *M. iners* PG30^T, *M. iowae* 695^T, *M. lipofaciens* R171^T, *M. meleagridis* 17529^T, *M. pullorum* CKK ^{T}, *M. sturni* UCMF ^{T} and *M. synoviae* WVU 1853^T. Information about the source of the 21 Ms field isolates used in this study is given in Table 1.

The mycoplasmas were cultivated in mycoplasma broth and plates (Bradbury, 1977) at 37 °C for between one and five days, in a CO_2 rich atmosphere. They were cloned once by filtration and the identity of all cultures was confirmed by the indirect fluorescent antibody test (Rosendal and Black, 1972).

Ten swab samples (Table 1) were also included. These were stored samples derived from tracheal swabs that had been submitted earlier for Ms isolation and which were culturally positive.

2.2. Sample preparation

Mycoplasma broth cultures (1.5 ml) in the exponential growth phase were centrifuged at 12,000 × *g* for 10 min. The pellet was washed twice in sterile PBS and resuspended in 200 μl sterile water (Sigma) by vigorous vortex mixing. A 25% suspension of Chelex 100 (Biorad) (200 μl) was added and vortex mixed again. The suspension was incubated at 56 °C for 30 min, and remixed. After placing in boiling water for 8 min, the mixture was cooled on ice, vortex mixed again, and centrifuged at 12,000 × *g* for 5 min. The supernatant fluid was transferred to a fresh tube. This solution containing the DNA was stored at −80 °C until use.

For sample preparation from swabs, each swab was suspended in 2 ml of mycoplasma broth and then 500 μl of this broth was then centrifuged after incubation at 37 °C for 1 h. The pellet was washed once in 500 μl of phosphate buffered saline (PBS) and then resuspended in 20 μl of PBS. Samples were heated at 95 °C for 2 min and then frozen until use.

2.3. DNA measurement

The DNA of the Ms strains (Table 1) was quantified with a fluorometer (DyNA Quant^{TM} 200, Hoefer, Pharmacia Biotech Inc.) following the manufacturer's instructions.

2.4. Primer design and use in amplification of the 16S-23S ISR

Ms forward primers (Ms1F and Ms2F) targeting the ISR were designed manually based on the avian *Mycoplasma* ISR sequences (GenBank accession numbers AJ780982–AJ781002, AB98503 and AB098504). From these sequences the two Ms species-specific regions were identified. Gene Runner