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A simplified duplex real-time PCR incorporating TaqMan minor groove binder (MGB) probes and an exogenous internal positive control for the simultaneous detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* cultures

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ABSTRACT: *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) are the most pathogenic and economically important mycoplasma pathogens that infect chickens. The development of rapid and innovative molecular diagnostic techniques is of pivotal importance for their effective control. The aim of the present study was to develop a novel duplex TaqMan real-time PCR assay for the simultaneous detection of MG and MS. This duplex real-time PCR assay incorporates TaqMan (FAM/NED) labelled minor groove binder (MGB) probes that target the cytadhesin encoding surface protein (*mgc2*) gene and the haemagglutinin surface protein (*vlhA*) gene of MG and MS, respectively. The assay also contained a TaqMan exogenous internal positive control (Exo IPC), to avoid false negative results that might happen due to failure in DNA extraction/PCR inhibition. The TaqMan MGB probe-based duplex RT-PCR incorporating Exo IPC was then applied to DNA from culture isolates for the simultaneous detection of MG (*mgc2* gene) and MS (*vlhA* gene). For duplex RT-PCR the sensitivity recorded was 10^{-3} CFU/ml and 10^{-2} CFU/ml for MG and MS template DNA, respectively. The specificity of the real-time PCR assay was 100% for MG- and MS-specific probes in detecting both single as well as double infections. In conclusion, the use of TaqMan MGB probes for the detection of *mgc2* and *vlhA* genes confers extra specificity and the incorporation of Exo IPC simplifies the assay, allowing the detection of double infections with low-copy target DNAs.

Keywords: avian *Mycoplasma* spp.; duplex real-time PCR; MGB probe; *mgc2* gene; *vlhA* gene; internal positive control

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are the most pathogenic and economically significant mycoplasma pathogens that infect chickens. The three major approaches currently used for the diagnosis of avian mycoplasmas are isolation and identification, detection of antibodies and molecular detection of the organisms by polymerase chain reaction (Raviv and Kleven 2009). PCR based on detection of *mgc2*-cytadhesin encoding surface protein gene for MG (Garcia et al. 2005), and *vlhA*-haemagglutinin surface protein gene of *M. synoviae* (Hong et al. 2004) are the most widely used methods for the detection, typing and determination of the source of infection. Real-time PCR (RT-PCR) avoids the need for post amplification processing, which saves time and labour compared to conventional PCR methods (Kawahara et al. 2008). TaqMan RT-PCR is a simpler method on which to base a multiplex PCR assay to detect mixed infections in a single PCR reaction. So far, only a limited number of TaqMan based diagnostic RT-PCR assays have been reported that target the *mgc2* gene (Grodio et al. 2008; Raviv and Kleven 2009; Sprygin et al. 2010) for detection of MG, and the *vlhA* gene for detection of MS. The duplex RT-PCR-based detection of MG and MS using the

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Table 1. Available gene-targeted duplex real-time PCR assays developed for the detection of *M. gallisepticum* and *M. synoviae*

Type of PCR	Target gene	PCR format	Organisms	Sensitivity for *M. gallisepticum*/*M. synoviae*	Reference
SYBR green real-time PCR	*16SrRNA*	D	*M. gallisepticum* and *M. synoviae*	27/28 DNA copies per reaction	Jarquin et al. 2009
TaqMan real-time PCR with IC (avian reo virus *S3* gene)	*mgc2/vlhA*	D		7/1 CFU equivalent/ml or 34 and 29 DNA copies per reaction	Sprygin et al. 2010
This study TaqMan real-time PCR with simplified commercially available TaqMan exogenous IC	*mgc2/vlhA*	D		$10^{-3}/10^{-2}$ CFU/ml (this study)	present study

D = duplex format, IC = internal control

TaqMan method has only been reported by Sprygin et al. (2010) as shown in Table 1. To increase the specificity of RT-PCR procedures, TaqMan minor groove binder (MGB™) probes are preferred because their small size enables binding in the minor groove of double-stranded DNA, thus stabilising the duplex, which results in high melting temperature (Tm) values (Kutyavin et al. 2000; Guo et al. 2009), and greater accuracy in measurements because of low background fluorescence (Farkas et al. 2009). Moreover, according to the requirements of the Office International des Epizooties (OIE), each PCR assay must incorporate an internal control (IC) (OIE 2008). The avian mycoplasma RT-PCR assays previously described by Grodio et al. (2008) and Sprygin et al. (2010) contained ICs. This study was aimed at developing a duplex TaqMan MGB-probe based RT-PCR, incorporating a TaqMan exogenous internal positive control (TaqMan® Exo IPC) for simultaneous detection of MG and MS. We propose that the developed assay will serve as a simplified and improved molecular detection method compared to the previously developed monoplex or duplex TaqMan RT-PCR methods for MG and MS.

MATERIAL AND METHODS

Bacterial Isolates and DNA extraction. Different MG strains (A5969, F, ts-11, 6/85) and MS strains (WVU 1853, F10-2AS, MS-H) were obtained from the Poultry Diagnostic and Research Center (PDRC), University of Georgia, Athens, USA and Charles River SPAFAS Inc., Storrs, CT, USA as detailed in Table 2. Local field isolates of MG (n = 4) and MS (n = 4) were isolated from chicken tracheal swab samples collected from the Faisalabad district, Pakistan. The tracheal swabs were subsequently inoculated on modified Frey's medium as formulated by Kleven (1998). Cultures were lysed by adding sodium dodecyl sulphate (SDS) at a final concentration of 1% (w/v). The DNA from avian mycoplasma cultures was extracted and purified using a phenol-chloroform extraction method as described by Wang et al. (1997). Concentration and purity of DNA was determined using a spectrophotometer (NanoDrop Model ND-1000, USA). The template DNA were mixed in various combinations in such a way that each reaction well received DNA from one field isolate of MG (1×10^4 CFU/ml) and one reference strain of MS (1×10^4 CFU/ml) and vice versa.

Synthesis of Primers and MGB probes. The primers and MGB probes for the *mgc2* gene of *M. gallisepticum* (GenBank accession No. FJ395202), and the *vlhA* gene of *M. synoviae* (GenBank accession No. FJ409871) were designed using ABI Primer Express Software Version 3.0 (Applied Biosystems, Foster city, CA, USA). The 5'-ends of TaqMan MGB probes were labelled with reporter dyes, FAM™ and NED™ for MG and MS, respectively, whereas the 3'-ends of both the probes contained a MGB/NFQ (Minor groove binder/non fluorescent quencher) as shown in Table 3. Both the TaqMan MGB probes were designed to have a Tm of 69 °C as per the manufacturer's instructions. The specificity of primers and probes was tested in BLASTn analysis.

Preparation of Duplex real-time PCR reaction mixture. The PCR reaction mixture was prepared in MicroAmp™ optical 96-well reaction plates (Applied Biosystems, Foster City, CA, USA), containing 1 µl of each template DNA for MG and MS (final concentrations of template DNA varied slightly), 1µM of each forward and reverse primer for MG and MS, 0.1µM of each TaqMan MGB probe

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Table 2. Detailed description of reference cultures of *Mycoplasma gallisepticum*, *M. synoviae* and *M. meleagridis* used in the study

Cultures*	Strain	Isolate No.	Source	Inoculation medium
M. gallisepticum	ts-11	–	PDRC	Frey's
	F	K810	PDRC	
	6/85	K3254	PDRC	
	A5969	–	PDRC	
	A5969	–	SPAFAS	
M. synoviae	WVU 1853	–	PDRC	Frey's
	WVU 1853	–	SPAFAS	
	F10-2AS	K758	PDRC	
	MS-H	K3928	PDRC	
M. meleagridis	E-2	–	PDRC	M-ORT
	E-2	K886	PDRC	

*avian Mycoplasma cultures were obtained from the Poultry Diagnostic and Research Center (PDRC), University of Georgia, Athens, USA and Charles River SPAFAS Inc., Storrs, CT, USA

for MG and MS, 200nM of 10 × TaqMan Exo IPC mix, 20nM of 50 × TaqMan Exo IPC DNA (TaqMan Exo IPC) and TaqMan Gene Expression master mix (Applied Biosystems, USA), in a total volume of 50 µl. The reproducibility of the TaqMan RT-PCR for detection of MG and MS was determined by carrying out two independent PCR assays which incorporated genomic template DNA extracted from nine 10-fold serial dilutions (10^4–10^{-4} CFU/ml) each of MG (strain A5969) and MS (strain WVU1853), starting with a titre of 1×10^4CFU/ml in duplicate runs.

Thermal conditions for duplex real-time PCR and data analysis. Amplifications were performed on an ABI-7500 real-time PCR system (Applied Biosystems, Foster city, CA, USA). The thermal cycling consisted of 50 °C for 2 min (for Uracil-DNA Glycosylase (UDG) optimisation) and 95 °C for 10 min (for AmpliTaq Gold UP activation) followed by 40 cycles with every denaturation step at 95 °C for 15 s. Annealing and extension were performed at 60 °C for 60 s. Data were analysed using ABI sequence detection system (SDS) software (version 1.4). Amplification results were expressed by plotting Delta Rn (ΔRn) versus Cycle number for the interpretation of double infection (Long et al. 2008).

RESULTS

The amplification curves detected from twenty-four reaction wells of the MicroAmp™ plate for simultaneous detection of MG and MS target DNA are depicted in Figure 1. The reproducibility results

Table 3. Primers and TaqMan MGB probes used in the duplex real-time PCR assay

Target spp.	Target gene	Primers/TaqMan MGB probes sequence	GenBank accession No.	Tm (°C)
M. gallisepticum	*mgc2*-F	5'-CAAGACCAAACTTCCCTAAC-3'	FJ395202	50
	mgc2-R	5'-CAGGTTGTGGTCTGAAACC-3'		52
	mgc2-probe	5'-[a]FAM-AAATGCCTAATATGAATCAACCA-MGB/NFQ-3'		69
M. synoviae	*vlhA*-F	5'-CCCAGGAGGTGGTACAGTTGAC-3'	FJ409871	59
	vlhA-R	5'-CATCAATAGCGGTTTTAGCTTCTG-3'		58
	vlhA-probe	5'-[b]NED-CTGTAGAGGCTGCTAAA-MGB/NFQ-3'		69

[a]FAM = TaqMan fluorescent dye 6-carboxyfluorescein; (TaqMan probe)

[b]NED = 7, 8'-benzo-5'-fluoro-2', 4, 7-trichloro-5 carboxyfluorescein; (TaqMan probe)

Tm = melting temperature; MGB/NFQ: minor groove binder/non-fluorescent quencher

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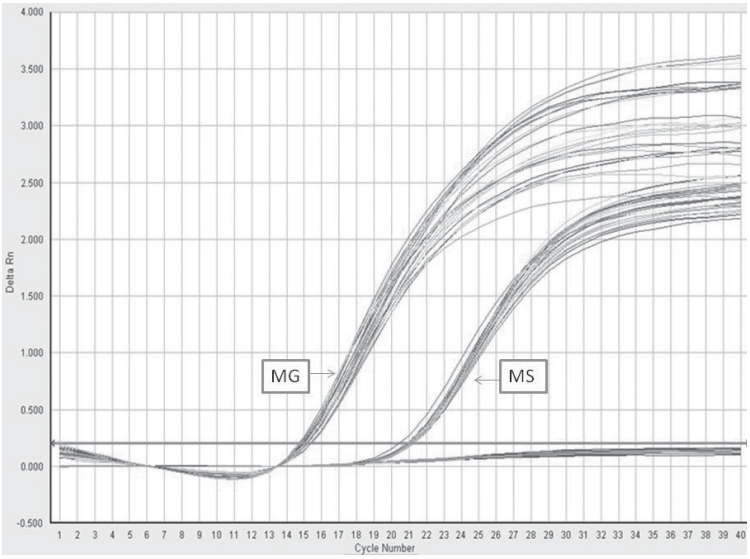

Figure 1. TaqMan duplex real-time PCR showing amplification curves (linear view) for simultaneous detection of *M. gallisepticum* (MG) and *M. synoviae* (MS). Only MG-MS duplex positive samples (n = 24) are shown in the figure. Mean Cycle threshold (Ct) values shown are 15.5 for MG and 20.5 for MS

are shown in the form of Ct values obtained from nine serial 10-fold dilutions in duplicates for MG (Table 4) and MS (Table 4) starting with a titre of 1×10^4cfu/ml.

For duplex RT-PCR, the sensitivity recoded was 10^{-3} CFU/ml and 10^{-2} CFU/ml for MG and MS template DNA, respectively. These DNA quantifications were made on the lowest dilutions with readable cut-off cycle threshold (Ct) values of 37.20 and 36.46 for MG and MS, respectively. The specificity of the real-time PCR assay was 100% for MG- and MS-specific probes in detecting both single (Figure 2) as well as double infections (Figure 1).

DISCUSSION

In the present study, a lower detection sensitivity (as indicated by higher Ct values) was observed with the TaqMan NED™ reporter dye as compared to the TaqMan FAM™. The higher Ct values might be due to the fact that the results documented for FAM™ have been reported to be more robust that those generated using the NED™ reporter dye (Anonymous 2013). Furthermore, the low detection value of NED™ as a reporter dye, also corroborates our past experience with MS-specific probe detection using FAM™ labelling in a single target (monoplex) TaqMan MGB real-time PCR reaction, which revealed lower Ct values (high levels of detection) for the same probe (Ehtisham-ul-Haque 2010; Ehtisham-ul-Haque et al. 2011). Therefore, a duplex RT-PCR assay was developed with combinations of FAM™- and NED™-labelled probes where a TaqMan VIC® labelled probe served as an internal positive control (IPC). Recently, a duplex TaqMan labelled probe-based RT-PCR assay for detection of MG (*mgc2* gene) and MS (*vlhA* gene) reported by Sprygin et al. (2010) utilised a black hole quencher (BHQ) as a non-fluorescent quencher, attached to the middle of probe. In contrast, the present method incorporated a non-fluorescent-quencher (NFQ) that combined with the MGB moiety at the 3'

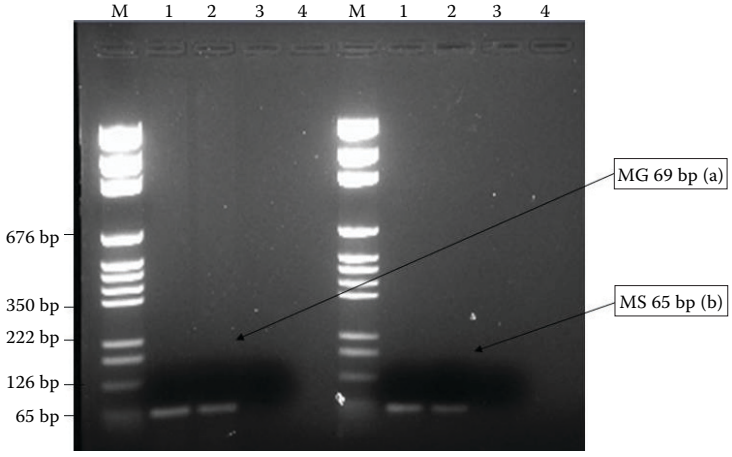

Figure 2. Amplicon size confirmation after TaqMan real-time PCR amplification showing monoplex detection of (a) *M. gallisepticum* (lane 1–2a) as a 69 bp amplicon and (b) *M. synoviae* lane (1–2b) as a 65 bp amplicon. Lane 3 = *Mycoplasma meleagridis* (MM); lane 4 = no template control (NTC); lane M = Bench Top pGEM DNA marker G7521 (Promega, Madison, WI, USA)

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Table 4. Reproducibility of the TaqMan RT-PCR for *M. gallisepticum* and for detection of *M. synoviae* showing Ct values obtained from two independent PCR assays

Task	Average Ct	Dilution of genomic DNA	Sensitivity (CFU/ml)
M. gallisepticum	20.66	10^{-1}	10^{4}
	21.19	10^{-2}	10^{3}
	22.53	10^{-3}	10^{2}
	23.67	10^{-4}	10^{1}
	25.37	10^{-5}	10^{0}
	29.30	10^{-6}	10^{-1}
	33.82	10^{-7}	10^{-2}
	37.20	10^{-8}	10^{-3}
	NR	10^{-9}	10^{-4}
M. synoviae	17.69	10^{-1}	10^{4}
	22.79	10^{-2}	10^{3}
	23.76	10^{-3}	10^{2}
	27.62	10^{-4}	10^{1}
	29.23	10^{-5}	10^{0}
	33.09	10^{-6}	10^{-1}
	36.46	10^{-7}	10^{-2}
	NR	10^{-8}	10^{-3}
	NR	10^{-9}	10^{-4}

NR = no Ct value recorded

end. The MGB probe with NFQ resulted in increased specificity in the detection of double infections by reducing the background fluorescence that might arise through the use of fluorescent quenchers (Farkas et al. 2009). Recently, Fraga et al. (2013) observed a problem in specificity when using conserved 16S*rRNA* gene primers for MS which detected *Mycoplasma cloacale* as well as MG and MS mixed infection in multiplex RT-PCR. Such non-specificity issues have not been reported for species-specific (*mgc2* and *vlhA* genes) primers/probes (Raviv and Kleven 2009; Sprygin et al. 2010). We determined specificity in the present study using *in silico* BLASTn analysis. Furthermore, the method described by Sprygin et al. (2010) incorporated avian Reo virus (*S3* gene) as an internal control (IC) in a Real-time PCR assay (Table 1). In contrast, we successfully incorporated commercially available (ready to use) TaqMan® Exo IPC to avoid false negative results that might happen due to failure in DNA extraction/PCR inhibition. By using the TaqMan® Exo IPC reagents, low-copy target DNAs were amplified in the reaction tube with the Exo IPC. Although the target and IPC DNAs differed in initial copy number, the amplification efficiency of the target reaction was not compromised. This was achieved by standardising the concentration of IPC primers in the PCR reaction.

The present study demonstrates that the use of commercially available Exo IPC simplifies the incorporation of internal controls in RT-PCR diagnostic assays. In conclusion, the TaqMan MGB probe-based duplex RT-PCR developed here for simultaneous detection of MG (*mgc2* gene) and MS (*vlhA gene*) from culture isolates, which also incorporates TaqMan Exo IPC, represents a simple, robust and more specific alternative to previously described methods. We envision that the assay will be of particular use to high-throughput diagnostic laboratories. Further studies are needed to validate the ability of the assay to detect MG and MS infections directly from clinical specimens without the need for bacterial culture.

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