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SHORT COMMUNICATION

Gene Targeted Sequencing Analysis of *Mycoplasma gallisepticum* Strains in Poultry Flocks from Middle East and South Asia

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ABSTRACT

The aim of this study was to attempt the molecular characterization of *Mycoplasma gallisepticum* (MG) strains detected in chicken flocks from United Arab Emirates (UAE), Saudi Arabia (SAU) and Sri Lanka (SL). Between 2012 and 2015, 55 samples from one layer flock from UAE, three breeder flocks from SAU and one broiler flock from SL were screened for MG using a pr-*mgc*2 PCR; the positive samples were characterized by the gene-targeted sequencing (GTS) analysis. BLAST comparisons, followed by phylogenetic analysis, was carried out to compare the obtained sequences against published vaccine and field strain sequences. Fifty of the fifty-five (91%) examined samples, were MG positive by pr-*mgc*2 PCR. The sequences detected in the samples from ts-11 vaccinated breeders from SAU showed 100% identity with the ts-11 vaccine sequences. The sequences detected in the samples from layers in UAE, were attributed to field strains, clustering together with Israeli MG strains. The sequences obtained from the samples from Sri Lanka broilers clustered separately from USA, Israeli, Australian, vaccine and reference strains. A potential route of MG field infection to the examined flocks may have been through the movement of birds, either directly (flock-to-flock transmission) or indirectly (through wild bird transmission). To our knowledge, this study reports the first molecular characterization by gene-targeted sequencing (GTS) analysis of MG strains from commercial poultry in SAU, UAE and SL.

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INTRODUCTION

Among Mycoplasma species, *Mycoplasma gallisepticum* (MG) is an economically significant pathogen that can cause infectious sinusitis in turkeys and chronic respiratory disease in chickens, leading to serious economic losses in poultry industry in terms of reduced feed efficiency, decreased growth and lower egg production. In addition, the carcasses of birds sent to slaughter may be downgraded (www.cfsph.iastate.edu 2018). Common approaches to prevent MG infections consist of maintaining infection-free breeder flocks through the use of strict biosecurity measures, continuous monitoring and vaccination in areas where complete eradication is difficult to attain (Raviv and Ley, 2013). Early detection of MG is essential to implement the appropriate measures required to prevent and manage the spread of infection. In this regard, molecular methods are largely used instead of culture, taking into account the slow mycoplasma growth and the frequent overgrowth of other mycoplasma species such as *Mycoplasma gallinarum* and *Mycoplasma gallinaceum*. Since the development and application of live MG vaccines, several molecular techniques for MG strain differentiation have been reported, including restriction fragment length polymorphism, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism, sequencing of the 16S and 23S rRNA intergenic spacer region (IGSR), and recently *vlhA* and *pvpA* gene-based real time PCRs or a combination of PCRs targeting *vlhA*3.05, *mg0359* and *vlhA*3.04a genes. A gene-targeted sequencing (GTS) analysis targeting portions of putative cytadhesin genes (*pvpA*, *gapA* and *mgc2*) and an uncharacterized hypothetical surface lipoprotein-encoding gene, designated coding DNA sequence (CDS) MGA_0319, has been reported as an accurate and reproducible method of typing MG strains (Ferguson *et al*., 2005). A major benefit of this approach is that the gene-targeted sequencing (GTS)

method allows the development of a reference database and standardized global comparisons between laboratories. The aim of this study was to characterize circulating MG strains using the gene-targeted sequencing (GTS) analysis for samples derived from different farms in different countries, and to cross-compare the results against available sequences in GenBank.

MATERIALS AND METHODS

Between 2012 and 2015, 55 samples from United Arab Emirates (UAE- layer - flock A), Saudi Arabia (SAU-breeder - flocks B, C and D) and Sri Lanka (SL-broiler - flock E) were submitted to the University of Liverpool for MG molecular testing. Birds from flocks A, B and C were sampled at two separate time points. The 55 submitted samples consisted of 40 tracheal swabs (flocks B, C and D) and 15 tissue samples (trachea, lung, turbinates and kidney) embedded onto Flinders Technology Association (FTA) cards (flock A and E) (Table 1). Clinical signs attributable to MG infection were reported in flock A and E prior to sampling. The breeder and layer flocks were reported to have received the live ts-11 MG vaccine.

Swabs or individual circles from FTA card were placed into 600 µl or 1.5 ml of working solution D (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1M 2-mercaptoethanol) (Chomczynski and Sacchi, 2006) respectively, then stored at -20°C for a minimum of three hours. DNA was extracted using the DNA Mini kit (Qiagen, Germany) according to manufacturer's instructions and stored at -20°C until use.

The extracted DNAs were first analyzed using a PCR assay targeting a 237-303 bp fragment of the proline-rich domain of the *mgc*2 adhesin-encoding gene (pr-*mgc2*) of MG (Moscoso *et al*., 2004). Pr-*mgc2* positive DNA samples were further characterized by GTS analysis (Ferguson *et al*., 2005), targeting the following genes: MGA_0319 (590 bp), *mgc*2 (824 bp), *gapA* (332 bp) and *pvpA* (702 bp). A single sample was selected for each sample point (Table 1) for molecular characterization. A modified forward pvpA3F (5'-GGYAGTCCTAAGTT ATTWGGTC-3') (Liu *et al*., 2001) primer was used in substitution for *pvpA* amplification (497 bp), to improve the success of detection.

Pr-*mgc*2 and all targeted gene (MGA_0319, *mgc*2, *gapA* and *pvpA*) amplicons were submitted for commercial bi-directional sequencing (Macrogen, Spain). Each sequence was edited and assembled using Bioedit, then BLAST comparisons were made with corresponding MG sequences available in GenBank (NCBI). Multigene sequences (*mgc*2/*pvpA*/MGA_0319/*gapA*) were aligned using Clustal V, concatenated and a Neighbor-Joining dendrogram was constructed in MEGA6 (Tamura *et al*., 2013) including field and vaccine MG strains retrieved from GenBank (Table 2). Bootstrap values, obtained with 1000 replicates, were considered significant when >70.

RESULTS AND DISCUSSION

The pr-*mgc*2 PCR showed 50 of 55 (91%) positive samples (Table 1). Pr-*mgc*2 fragments were identical for all the samples of the same geographical origin but differed from each other forming three distinct clusters according to the country of origin (Fig. 1).

The gene-targeted sequencing (GTS) analysis was performed on 1/2 representative strains from each sampled flock and age of sampling. Strains included in the analysis were UAE/10/CK/12/44wks and UAE/10/CK/12/64wks (flock A), SAU/19/CK/15/19wks and SAU/19/CK/15/32wks (flock B), SAU/25/CK/15/16wks and SAU/25/CK/24wks (flock C), SAU/28/CK/15/32wks (flock D) and SL/1/CK/15 (flock E).

MGA_0319, *mgc2*, *gapA* and *pvpA* gene sequences were obtained from all strains with the exception of UAE/10/CK/12/44wks and UAE/10/CK/12/64wks for which the sequencing of the *pvpA* gene was unsuccessful. The gene-targeted sequencing analysis confirmed the clustering of sequences according to the country of origin. Based on nucleotide identity, one representative sequence for country was submitted to GenBank. The following accession numbers were assigned: MK217482, MK217478, MK114557 (*mgc*2, *gapA*, MGA_0319 UAE/10/CK/12/44wk MG strain), MK217480, MK217477, MK217479, MK217481 (*mgc*2, *gapA*, MGA_0319, *pvpA* SAU/28/CK/15/32wk MG strain), MK036426, MK036427, MK036428, MK036429 (*mgc*2, *gapA*, MGA_0319, *pvpA* SL/1/CK/15 MG strain).

The five SAU strains, all from ts-11 vaccinated breeders, clustered with the ts-11 vaccine strain (Fig. 2), demonstrating 100% nucleotide sequence identity in all analyzed genes to each other and the ts-11 vaccine. Both an increase in vaccine virulence, and vertical transmission of ts-11 vaccine have been previously reported (Armour and Ferguson-Noel, 2015). However, the absence of typical MG clinical signs suggested that our detections were not likely to be revertant. A complete genome sequencing of the strains obtained in this study could better explain the relationship between our detections and the ts-11 vaccine.

Despite the sampled layer flocks from the UAE being vaccinated with ts-11, the detected strains were potentially field strains, due to their low nucleotide identity when compared with the vaccine strains (97.1% in MGA_0319, 96.5% in *mgc2* and 97.8% in *gapA*) and forming a distinct cluster with Israeli MG field strains (OR-2, BRT-14) (Fig. 2). In addition, the strains UAE/10/CK/12/44wks and UAE/10/CK/12/64wks were detected from kidney of layer birds affected by clinical signs. Even if MG field strains

Table 1: Vaccination history, sampling time and PCR results of investigated flocks

Year of sampling	Country	Flock	MG vaccination programme (5 week)	Age at sampling (week)	PCR for pr-*mgc2* (No. pos/No. tested)
2012	United Arab Emirates	A (layer)	ts-11	44	tissues (4/4)
				64	tissues (4/4)
2015	Saudi Arabia	B (breeder)	ts-11	19	tracheal swabs (4/6)
				32	tracheal swabs (1/1)
2015	Saudi Arabia	C (breeder)	ts-11	16	tracheal swabs (7/10)
				24	tracheal swabs (11/11)
2015	Saudi Arabia	D (breeder)	ts-11	32	tracheal swabs (12/12)
2015	Sri Lanka	E (broiler)	Not vaccinated	Not known	tissues (7/7)

Table 2: Published MG sequences used in the gene-targeted sequencing (GTS) analysis

Field strain	Country	GenBank accession number			
		mgc2	*gapA*	MGA_0319	*pvpA*
S6	USA	KY421064.1	JQ770168.1	AY556073.1	EU847585.1
R	USA	AY556228.1	AY556150.1	AY556072.1	AY556306.1
OR-2	Israel	AY556296.1	AY556219.1	AY556140.1	AY556373.1
BRT14	Israel	AY556291.1	AY556214.1	AY556136.1	AY556368.1
AU96022CK96	Australia	AY556301.1	AY556224.1	AY556145.1	AY556378.1
K503	USA	AY556234.1	AY556156.1	AY556078.1	AY556310.1
A5969-CK55	USA	AY556227.1	AY556149.1	AY556071.1	AY556305.1
Vaccine strain					
TS-11		AY556232.1	AY556154.1	AY556076.1	AY556382.1
F		AY556230.1	AY556152.1	AY556074.1	JN001169.1
6/85		KP318741.1	JQ770170.1	/	KP881243.1

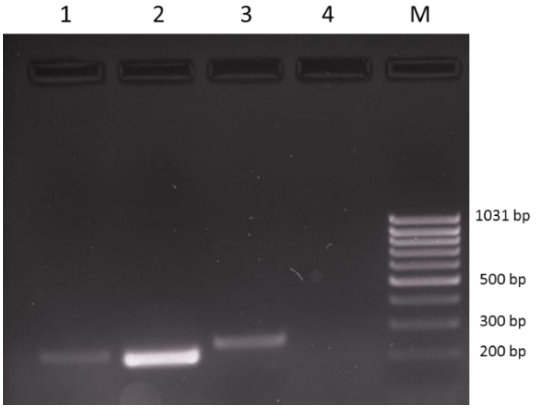

Fig. 1: Pr-*mgc*2 PCR results. Lane 1: representative amplicon from United Arab Emirates samples. Lane 2: representative amplicon from Saudi Arabia samples. Lane 3: representative amplicon from Sri Lanka samples. Lane 4: negative control. M: MassRuler Low Range DNA Ladder, Promega.

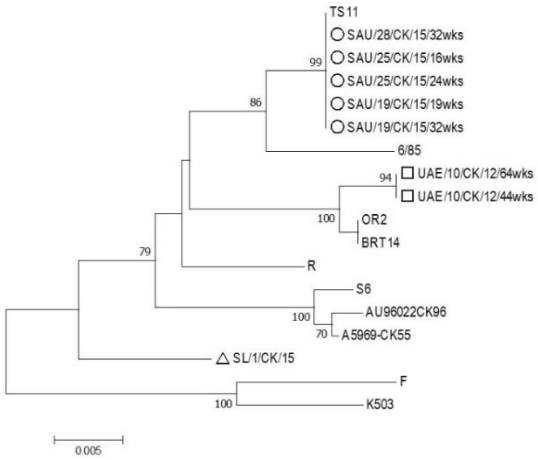

Fig. 2: Dendogram based on the alignment of nucleotide sequences of MGA_0319, *mgc*2, *gapA* and *pvpA* genes of MG strains detected in the study and USA, Israeli, Australian, vaccine and reference strains. Only bootstrap values >70 were reported. The MG sequences were labelled with a square (UAE), a circle (SAU) or a triangle (SL), according to the country of origin. UAE/10/ck/12/44wk and UAE/10/ck/12/64wk sequences were lacking of the *pvp*A gene.

can be isolated from the respiratory tracts of protected chickens (Khalifa *et al*., 2014), as the vaccine prevents invasion of other tissues, detection from visceral tissue is considered highly indicative of clinical disease (Bíró *et al*., 2005). Moreover, it should be noted that the birds were sampled at 44 and 64 wks of age (Table 1) and these time points fall close or outside of the ts-11 vaccine protection window (up to 40 wks post-vaccination as reported from manufacturer's instructions).

MG circulation was reported in both Sri Lanka breeder and broiler chickens (Weerasooriya *et al*., 2017). In this study, SL/1/CK/15 MG strain, originating from Sri Lanka broilers affected by respiratory disease, was considered as a potential field strain, as it clustered separately from USA, Israeli, Australian, vaccine and reference strains (Fig. 2).

A potential route of MG field infection to the examined may have been through the movement of birds, either directly (flock to flock transmission) or indirectly (through wild bird transmission) (Raviv and Ley, 2013). Moreover, based on the phylogenetic analysis, UAE strains had close genetic relationship with Israeli MG strains, suggesting the epidemio-geographical relationship between these strains. To our knowledge, this study reports the first molecular characterization by gene-targeted sequencing (GTS) analysis of MG strains from commercial poultry in SAU, UAE and SL.

Authors contribution: VF conceived and executed the experiment. CB and KG conceived and designed the experiment. EC reviewed the manuscript. ADF conceived the experiment and wrote the paper. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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