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MOLECULAR CHARACTERISATION OF *MYCOPLASMA GALLISEPTICUM MGC2* GENE FROM COMMERCIAL CHICKEN ISOLATES

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SUMMARY

Mycoplasma gallisepticum (MG), an agent that causes chronic respiratory diseases in the avian, possesses a cytadhesin *mgc2* gene. *Mgc2* is a second cytadhesin-like protein, localised on the terminal bleb involved in the attachment of MG to host tissue. Five reference MG strains (S6, R, F, TS11 and 6/85) and 18 field isolates were extracted, sequenced and analysed, including another 30 published isolate sequences from U.S., Australia and Israel. The *mgc2* gene isolates exhibit high G + C base content of 45% due to the presence of high proline (14 to 16%) and glycine (13 to 14%) residues located at the two-third position of the carboxy terminal region. The Malaysian field isolates were divided into four categories: (i) 854 - 857 bp amplicon, (ii) 837 bp amplicon, (iii) 822 - 824 bp amplicon, and (iv) 791 bp amplicon, due to gene size polymorphism. Six field isolates (KPR44 L, KPR16W44 L, THNG8W L, AK2 VC, PF3H Br and PF7U Br) exhibited several mutations at the 3' region located at positions 166, 173, 195 and 202 a.a., which distinctively differ from other reference strains but appear identical to Israeli isolates. Other field isolates and certain published sequences were either totally similar or almost similar to MGS6, TS11and 6/85 strains.

Keywords: *Mycoplasma gallisepticum, mgc2* gene, molecular characterisation, commercial chickens

INTRODUCTION

Mycoplasma gallisepticum (MG) infection in poultry has become significantly important as the disease causes low production of chickens and eggs resulting in economic losses to the commercial poultry industry on a long term basis. *Mycoplasma gallisepticum* is known to infect mucosal surface of respiratory and urogenital tract and manifests a wide variety of clinical symptoms such as rales, coughing, sneezing, ocular and nasal discharge, decreased food consumption, decreased egg production and a poor hatchability rate (Bradbury, 2002; Nascimento *et al.*, 2005).

Of the 23 avian mycoplasmas known (Bradbury, 2002), MG is one of the pathogenic avian mycoplasmas that has capabilities to adhere to host target cell and mediate apoptosis, antigen mimicry and to vary phenotype at a high frequency (Simecka *et al.*, 1992; Nascimento *et al.*, 2005). Adhesion of MG to epithelial cells is facilitated by adhesion proteins on the specialised terminal ends of organelle located at the surface membrane (Razin and Jacob, 1992), namely, MGC1, MGC2, GapA and PvpA (Boguslavsky *et al.*, 2000; Goh *et al.*, 1998; Hnatow *et al.*, 1998; Keeler *et al.*, 1996). *Mgc2* is a second cytadhesin-like protein, localised to the terminal bleb involved in attachment of MG to host tissue. It contains 912-nucleotide that encodes a 32.6 kDa protein with a sequence homology of 31.4% and 40.9% towards *M. genitalium* P32 and *M. pneumonia* P30 cytadhesin, respectively. The proteins of *mgc2*, P30 and P32 proteins share 30 proline residues including consensus tryptophan residues at amino acid positions 62 in *mgc2*, 73 in P30 and 68 in P32. This indicates that mycoplasma from widely divergent hosts utilise homologous cytadhesin proteins for attachment to host tissue, which suggests the importance of these membrane proteins to successful exploitation of the host mucosal niche (Hnatow *et al.*, 1998).

Although few reports of *mgc2* gene characteristics have been published (Hnatow *et al.*, 1998; Ferguson *et al.*, 2005), no study has been conducted on the molecular basis of *mgc2* gene from Malaysian isolates. As the *mgc2* gene exhibits different mutational features in different environments, the present study explores variation features exhibited by the *mgc2* gene in clinical isolates of MG from Malaysia. A comparison with other reported strain sequences was also undertaken.

MATERIALS AND METHODS

Mycoplasma gallisepticum reference strains and isolates of *Mycoplasma gallisepticum* reference strains (S6, R, F, TS11 and 6/85) including 17 MG field isolates from Malaysia were obtained from Dr Tan Ching Giap from the Faculty of Veterinary Medicine, Universiti Putra Malaysia. A MG field isolate from commercial broiler

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chickens, namely PB14T Br, from a sample of this current study was also used. The isolation and purification of the strain and growth conditions have been described elsewhere (Yamamoto *et al.*, 1992). Eighteen partial *mgc2* gene sequences from the U.S., four from Australia and eight from Israel were extracted from PubMed GenBank sequence for comparison purposes.

Isolation of genomic DNA

One millilitre of MG culture was harvested and centrifuged at 14,000 rpm for 10 minutes. The supernatant was discarded, and the pellets were resuspended in 1 ml PBS 1x in Eppendorf tubes. The cell was lysed by adding 60 μl of 10% Sodium Duodecyl Sulphate (SDS) solution together with 1 μl of 50 μg/μl Proteinase K into the isolate solution, and then vortex mixed for 10 seconds. The tubes were incubated in a warm water bath at 65°C for 30 minutes and were shaken every 5 minutes for 5 seconds. The lysed samples were cooled down to -20°C for 10 minutes.

Proteins were separated from the mixture by adding 300 μl of Ammonia acetate 5M into each tube and the mixture was vortex mixed for 10 seconds. The debris was pelleted by centrifugation at 14,000 rpm, 4°C for 10 minutes. The supernatant was transferred into a new 1.5 ml microcentrifuge tube and the pellet was discarded. A volume of 550 μl of isopropanol was added to the recovered supernatant to precipitate the total nucleic acids. The tubes were inverted 30-40 times to mix the solution. The solution was centrifuged at 14,000 rpm at 4°C for 10 minutes to pellet the total nucleic acids. The isopropanol was slowly poured out from the tube. The nucleic acid pellet was then rinsed with 1 ml of 75% ethanol two times. The total nucleic acids were pelleted by centrifugation for 10 minutes at 14,000 rpm at 4°C. The ethanol was poured out slowly and the procedure was repeated once. The pellet was dried out in a laminar flow chamber. On drying out of the pellet, a volume of 30 μl of distilled deionised water was added into the tube and then stored in -20°C for further use.

PCR and oligonucleotides

The *mgc2* gene from MG strains was amplified by PCR. Reactions were carried out in 25 μl containing 2.0 μl DNA (10 ng/μl) template, 2.5 μl of 10x PCR buffer, 2.5 μl $MgCl_2$ (2.5 mM), 1.0 μl dNTP (0.4 mM), 0.5 μl primers (20 μM), 0.2 μl Taq DNA Polymerase (0.04 U/μl) and 15.8 μl sterilised deionised distilled water. PCR amplification was performed in a MyCycler® Thermal Cycler (Bio-Rad, Hercules, CA, USA). The initial PCR cycle was carried out using the following cycling parameters: initial denaturation of 94°C for 3 minutes, followed by 40 cycles of denaturation, annealing and extension at 94°C for 30 seconds, 58°C for 40 seconds and 72°C for 1 minute and 30 seconds, respectively. The final extension was carried out at 72°C for 5 minutes, followed by slow cooling at 10°C. The nucleotide sequence designated MGC2 1F: 5'GCT TTG TGT TCT CGG GTGCTA3' and MGC2 1R: 5' CGG TGG AAA ACC AGCTCT TG3' has been described previously (Steinlage *et al.*, 2003).

Sequence analysis

The PCR products for all 18 field isolates including MG laboratory reference strains (MGS6 and R) and vaccine strains (F, TS11 and 6/85) in this study were purified by DNA purification kit (GeneAll® Expin™ Gel SV) and directly send for sequencing to Macrogen Incorporation, Korea (Automatic Sequencer - ABI 3730xl DNA Analyser). To conduct sequence analysis of the *mgc2* gene, all strain sequences were truncated to obtain a common position within the coding sequence. The sequence of nucleotides and deduced amino acids were aligned using the BioEdit Sequence Alignment Editor. The phylogenetic tree was constructed by using the Neighbor-joining method, 1000 bootstrap in MEGA5 programmes, based on deduced amino acids sequences.

RESULTS

mgc2 gene PCR products

Field strains, vaccine strains and laboratory reference strains demonstrated different PCR amplicon sizes of the *mgc2* gene, within a range of 791 - 857 bp. Size variation of mgc2 PCR products among field and reference isolates could be divided into four categories: (i) 854 - 857 bp amplicon, (ii) 837 bp amplicon, (iii) 822 - 824 bp amplicon, and (iv) 791 bp amplicon. MG strain F produced the longest amplicon size of 857 bp. MG strain S6, TS11 and 13 field isolates produced an amplicon size of 854 bp, followed by one field isolate THNG8W_L at 837 bp. MG strain R and two field isolates, KWN2020_L and I44_VC, produced an amplicon size within the range of 822 – 824 bp. MG strain 6/85 and two field isolates, PB14T_Br and AK9_VC, produced the shortest amplicon size of 791 bp (Table 1).

Sequence analysis of mgc2 gene

Examination of the *mgc2* nucleotide sequence and of the deduced amino acid sequence revealed several notable features. Based on complete nucleotide sequence of the *mgc2* gene, the G + C base content of the *mgc2* gene was 45%, which is higher than the average G + C content for MG genome (32 to 36%) (Razin *et al.*, 1998). Sequence comparisons of *mgc2* gene among field isolates and main reference strain (S6) are listed in Table 2. All truncated sequences from field isolates, reference isolates and published *mgc2* gene sequences from U.S., Australia and Israeli isolates were aligned. Based on the deletion and mutation patterns of all the *mgc2* sequences, reference

Table 1: Size of *Mycoplasma gallisepticum mgc2* gene PCR product from reference and field isolates

Strain / Isolates	Source of isolates	Size of PCR product (bp)
F[1]	PPLO broth	857
S6[V]	PPLO broth	854
TS11[2]	PPLO broth	854
KWN2036 L[3]	Layer chicken	854
KPR44 L[3]	Layer chicken	854
KPR16W44 L[3]	Layer chicken	854
EES L[3]	Layer chicken	854
3B BB[3]	Breeder broiler	854
H21VT8 BB[3]	Breeder broiler	854
H21VT11 BB[3]	Breeder broiler	854
H269C2 BB[3]	Breeder broiler	854
H26SL2 BB[3]	Breeder broiler	854
I29 VC[3]	Village chicken	854
T25 VC[3]	Village chicken	854
A5 VC[3]	Village chicken	854
AK2 VC[3]	Village chicken	854
THNG8W L[3]	Layer chicken	837
R*	PPLO broth	824
KWN2020 L[3]	Layer chicken	823
I44 VC[3]	Village chicken	822
6/85	PPLO broth	791
AK9 VC[3]	Village chicken	791
PB14T Br	Broiler chicken	791

* Provided by Prof. Dr. S. H. Kleven, PDRC, University of Georgia, Athens, USA.
[V] Provided by Veterinary Research Institute, Ipoh, Perak, Malaysia.
[1] Obtained from Schering-Plough Animal Health, Omaha, NE.
[2] Obtained fromVAXSAFE MG TS-11, BIOPROPERTIES Pty. Ltd.
[3] Obtained from Dr. Tan Ching Giap, Faculty of Veterinary Medicine, UPM

strains were divided into five types: (i) laboratory strain S6, (ii) laboratory strain R, (iii) MG vaccine strain F, (iv) MG vaccine strain TS11, and (v) MG vaccine strain 6/85. No deletions were observed in sequences within the S6 strain group, TS11 strain group and other field isolates. Deletions were observed in isolate sequences within strain R group with 15 nt deletions each at two different positions, and 63 nt deletions in strain 6/85 group. However, insertion of nucleotide sequence was observed in MG vaccine strain F at position 481-483 nt.

Sequence similarity for deduced amino acids sequence among field isolates towards strain S6 was demonstrated by three isolate from layer chickens (KWN2036 L, KWN2020 L and EES L), five isolates from broiler breeder chickens (3B BB, H21VT8 BB, H21VT11 BB, H269C2 BB and H26SL2 BB) and three isolates from village chickens (I44 VC, I29 VC and T25 VC) which shared 100% amino acid sequence similarity. However, other field isolates including published reference isolates shared 85-99% amino acids sequence identity with strain S6 as shown in Table 2.

Amino acids (a.a) mutation of *mgc2* sequence in field and reference isolates were noticed as shown in Figure 1. Eight field isolates, namely KPR44 L, KPR16W44 L, THNG8W L, VPTIH6 PE, H6VTPI PE, AK2 VC, PF3H Br and PF7U Br showed several mutations at the 3' region located at positions 166, 173, 195 and 202 a.a., which distinctively differ from other reference strains and published isolate sequences. Other field isolates and certain published sequences were either totally similar or almost similar to MG S6, F, TS11, 6/85 strains; while other published isolate sequences, especially the sequences of isolates from Israel and the US were similar to house finch *mgc2* isolate HF51, and were significantly different from any reference strains sequences.

Mgc2 produced different gene sizes in certain strains that could be noted from both nucleotide (data not shown) and deduced amino acid sequences. MG strain F produced 285 a.a., the highest gene size, with one insertion of asparagine (N) at a.a. position 161. MG reference strain S6, including TS11, and other field isolates identical to both reference strains above, produced 206 a.a. gene size. Gene size of R and 685 strains were less compared to S6 strain with gene size of 198 a.a. and 185 a.a. due to deletions of eight a.a. at two different sites near 3' region and 21 a.a. near 5' region, respectively.

Table 2: Sequence analysis of the *mgc2* gene from reference strains and field isolates

Strain / Isolates	% sequence identity		Position of amino acid changes (based on aligned sequence)	Accession number
	Nucleotide (n.t.)	Amino acid (a.a.)		
S6	100	100	-	AY556229
TS11	97.3	96.1	64,65, 85, 107, 114,195,202.	AY556232
F	93.5	94.2	53,62,64,73,81,85,108,127,148,161,196,202.	AY556230
R	93.6	92.7	118,124,202.	AY556228
6/85	86.9	85.9	53,62,85,109,124,125,195,202.	AY556231
A5969	100	99	193.	AY556227
HF51	98.3	98	90,192,193,202.	AY556233
KWN2036 L	100	100	-	-
KWN2020 L	100	100	-	-
EES L	100	100	-	-
3B BB	100	100	-	-
H21VT8 BB	100	100	-	-
H21VT11 BB	100	100	-	-
H269C2 BB	100	100	-	-
H26SL2 BB	100	100	-	-
I44 VC	100	100	-	-
I29 VC	100	100	-	-
T25 VC	100	100	-	-
KPR44 L	98.5	98	166,173,195,202	-
KPR16W44 L	98.5	98	166,173,195,202	-
THNG8W L	98.5	98	166,173,195,202	-
AK2 VC	98.5	98	166,173,195,202	-
PF3H Br	98.5	98	166,173,195,202	-
PF7U Br	98.5	98	166,173,195,202	-
A5 VC	97.3	96.1	64,65,85,107,114,195,202	-
AK9 VC	86.9	85.9	53,62,85,109,124,125,195,202	-
PB14T Br	86.9	85.9	53,62,85,109,124,125,195,202	-
			118,124,193,202	AY556239
			.90,93,94,202	AY556238,-240, -273,-282.
			64,65,85,107,114,193,195,202	AY556239,-270, -274, -285.
			.53,62,85,109,124,125,193,195,202.	AY556272,-284, -289.
			53,62,64,73,81,108,127,139,148,	AY556234
US isolates (18)	86.9 – 98.5	84.9 - 99	153,161,176,177,193,195,202.	
			53,57,62,64,73,81,108,113,127, 141,148,161,193,195,196,202.	AY566235
			53,62,64,73,81,91,108,122, 123,127,148,161,193,199,202.	AY556236
			53,62,85,109,124,125,193,195, 202.	AY556241
			193.	AY556271
			53,62,85,109,124,125,193,195, 202.	AY556287
			64,65,85,107,114,193,195,202.	AY556299,-300,-304.
Australian isolates (4)	89.5 – 97.3	88.3 - 95.1	191, 193.	AY556301
Israeli isolates (8)	98.2 – 98.5	96.6 - 97	166,173,193,195,202.	AY556291-98

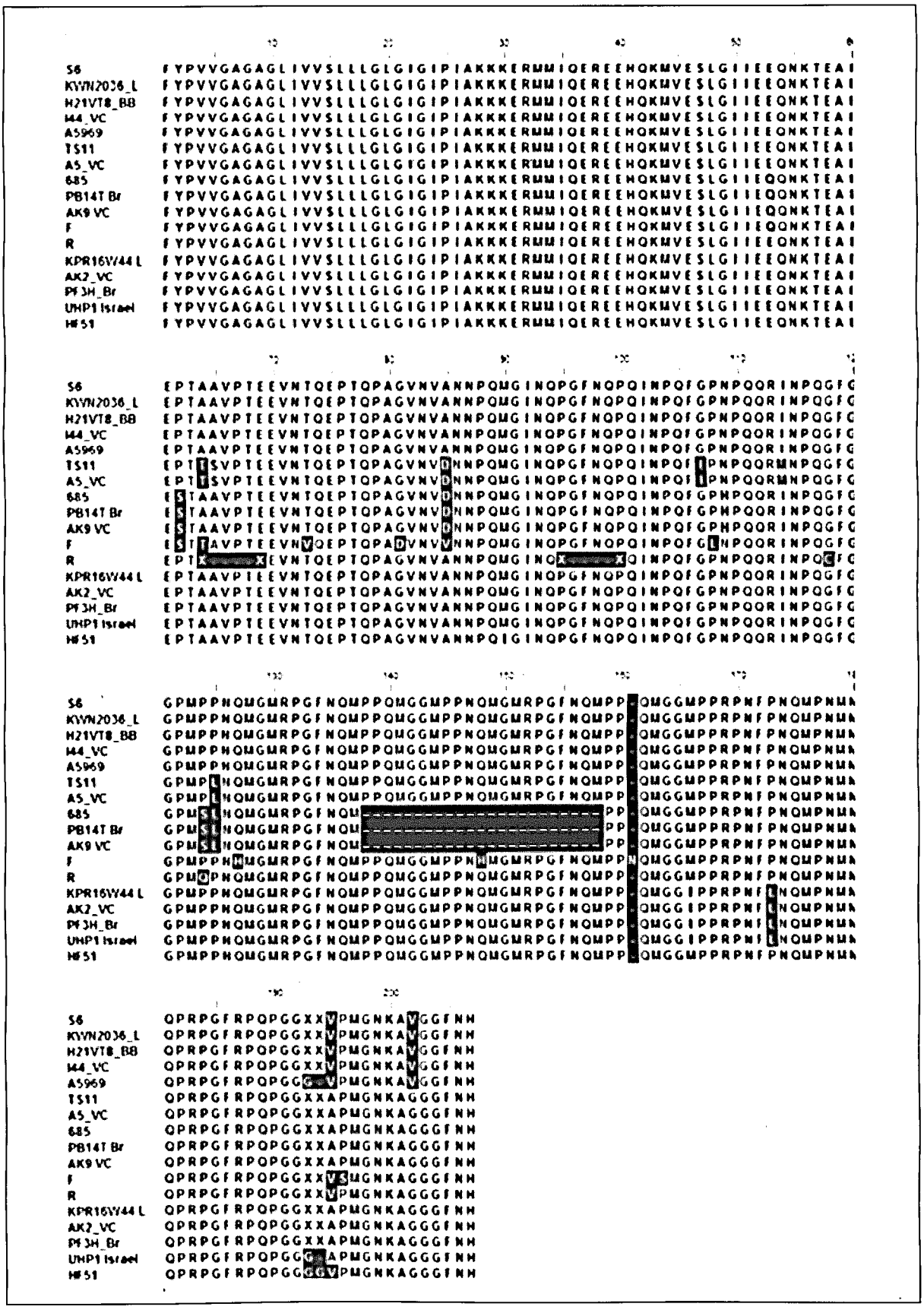

Figure 1: Comparison of the deduced amino acid sequences of mgc2 from *M. gallisepticum* reference strains, published and field isolates in 5' to 3' direction.

Note: Sequences shared by two types of amino acid are shaded in grey, while amino acid changes in sequences are shaded in black; dashes represent gaps.

Phylogenetic analysis of mgc2 isolates

Based on phylogenetic analysis of deduced amino acids of *mgc2* sequences in Figure 2, field isolates can be divided into four different groups. Eleven field isolates from layer, breeder broiler and village chickens were within the MGS6 strain group as shown in clade I. Six field isolates from layer, broiler and village chickens were within the same group with Israel isolates as shown in clade II. Two field isolates, namely PB14T and AK9, from broiler and village chickens respectively, were within the same group as 6/85 strain in clade III. Only one field isolate from village chicken, namely A5 VC, was within same group as TS11 strain in clade IV.

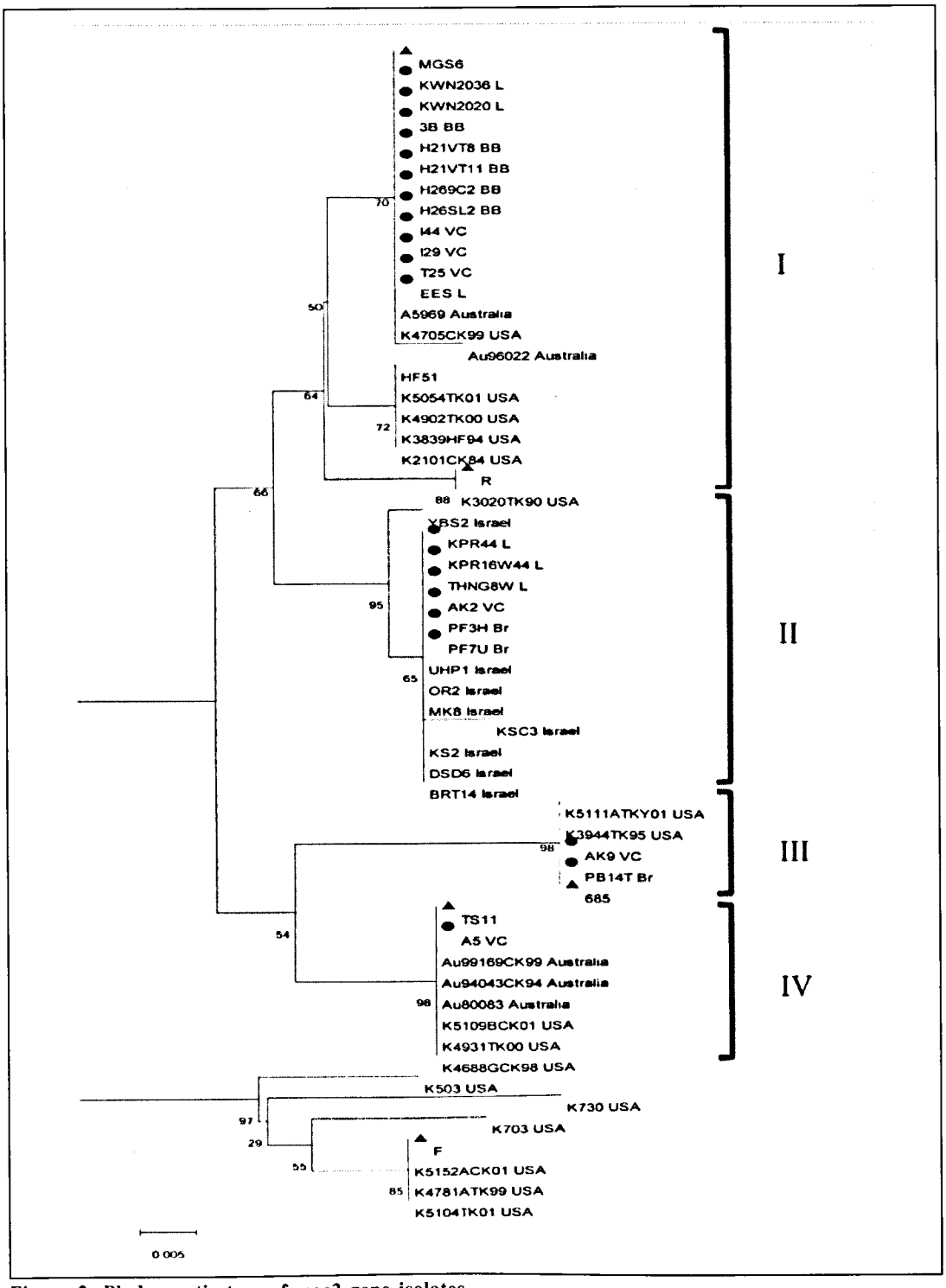

Figure 2: Phylogenetic tree of *mgc2* gene isolates

Note: All reference strains are triangle labelled, while the Malaysian field isolates from broiler, broiler breeder, layer and village chickens are circle labelled.

DISCUSSION

Genetic analysis in this study provides evidence that Malaysian MG field isolates from different type commercial chickens have similarities to reference strains and published isolates. The *mgc2* gene is a second cytadhesin protein that encodes a surface protein, known to play a role in the attachment process to host tissue (Hnatow *et al.*, 1998). The *mgc2* gene from Malaysian field isolates are characterised by high G+C contents in accordance to findings by Hnatow *et al.* (1998), despite a low G+C content for the entire genome as described by Razin *et al.* (1998). This can be attributed to the presence of high proline (14 to 16%) and glycine (13 to 14%) residues, located at the two-third position of the carboxy terminal region.

Gene size polymorphism was evident among *Mgc2* PCR products and gene sequences. Gene size polymorphism means different MG isolates produce different sizes of the particular gene within the limited range. Based on sequence analysis, *mgc2* gene variations in certain MG isolates were attributed to insertion of asparagine (N) amino acid in F strain sequence and deletion of sequence in strain R and 685. Thus, variation in gene size allowed the establishment of sequence clusters that included local isolates from known poultry outbreaks, isolates closely related to vaccine strains and isolates from reference strains. The identification of gene polymorphism in bacteria by nucleotide sequence analysis of genes encoding antigenic surface proteins has been proven useful in the surveillance of pathogenic bacteria (Byun *et al.*, 1999; Kotetishvili *et al.*, 2003). A previous study by Ferguson *et al.* (2005) has reported gene size polymorphism in the *mgc2* gene of US isolates in PCR products that range from 761bp, 824bp, 839bp and 854bp. Genotyping of MG strains through gene-targeted sequencing (GTS) can be achieved by using multiple gene sequence of surface-protein gene (*pvpA, mgc2, gapA* and *MGA_0319*). Since the surface protein is exposed to the environment, the *mgc2* gene may exhibit gene size polymorphism due to unknown environmental or climatic factors.

Some local isolates were close to the vaccine strains TS11 and 6/85, and reference strain S6. Whether these isolates are TS11 or 6/85-derived vaccine subpopulations, or isolates closely related to the vaccine and/or reference strains that evolved independently in the field is still not clear. Six Malaysian field isolates were identical to Israeli field isolates with 98.5 to 99% homology. The source of Israeli isolates has been described elsewhere (Ferguson *et al.*, 2005). It is not clearly understood whether the Malaysian MG field isolates evolved through mutation for adaptation that it can be identical to Israeli isolates or that the MG was derived from Israel through importation of broiler breeder parent stock from that country. Further analysis of complete field genome and genetic stability of live MG vaccines in the field is needed to precisely determine the relation of these vaccine-like isolates.

It is difficult to eradicate MG infection in commercial chicken flocks once an outbreak occurs. In Malaysia, immunisation with attenuated or live vaccine and treatment with antibacterial drugs are the main control measures employed in the commercial chicken sector. However, excessive and prolonged usage of vaccines poses a risk of virulence recovery and may cause MG outbreaks among vaccinated flocks. Therefore, a more reliable and reproducible detection method is crucial to identify MG strains and also to evaluate the efficacy of vaccine strains. In this study, several noteworthy features of Malaysian isolates deserve further attention particularly where the field isolated *mgc2* gene shared identical variations with the virulent strains S6, vaccine strains TS11 and 6/85 but exhibited a different variation from the published Israeli isolates. These results provide evidence that the *mgc2* gene can be used as a target for the establishment of real-time diagnosis of MG epidemics.

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