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Molecular Identification of Two Genetic Markers That Distinguish Between Pathogenic and Nonpathogenic Strains of *Mycoplasma gallisepticum*

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Abstract: A total of 571 *Mycoplasma gallisepticum* (MG) field isolates originated from progenies and commercial poultry farms in Malaysia and 7 reference and vaccine strains were characterized by amplification of selected gene target specific sequences to *MG pMGA* and *pvpA* genes using conventional PCR of sequence specific primers. A total of 281 MG positive field isolates out of 571 MG samples were detected with the primer targeted *pMGA* gene and a total of 188 MG positive field isolates out of 571 MG samples were detected with the primer targeted *pvpA* gene. Similar and identical banding pattern among MG isolates obtained from progenies samples however, there was a variable on the banding pattern among MG isolates obtained from commercial chickens using the agarose gel electrophoresis. The sequencing analysis results of MG based on selected genes targeted specific sequences were obtained. The genetic diversity of the *pMGA* and *pvpA* genes of MG field isolates detected in progenies and commercial chickens were investigated. The gene size variation patterns of the *pMGA* and *pvpA* genes among MG field isolates shared identical variations with the pathogenic reference and vaccine strains that is an insertion bp fragments by using the *pMGA* gene primer set and a deletion bp fragments by using the *pvpA* gene primer set. However, the gene size variation patterns are quite different from the variation pattern of the less pathogenic vaccine strain that can't be transmitted vertically. The polymorphism pattern of the primer for *pMGA* gene might be considered as a pathogenic vertical marker and the polymorphisms patterns of the two primers sets for both *pMGA* and *pvpA* genes might be useful for determining the two genetic potential pathogenic marker for MG infection that can differentiate between the highly and the less pathogenic MG isolates.

Key words: *Mycoplasma gallisepticum*, *pMGA* gene, *pvpA* gene, polymorphisms patterns, pathogenic marker, Malaysia

INTRODUCTION

Mycoplasma gallisepticum (MG) is a worldwide major problem in poultry industry causing chronic respiratory disease of chickens and turkeys. The surface proteins antigenic variation of MG allowed the host's immune response evation through the escape variants generation (Gorton and Geary, 1997; Glew *et al*., 2000). The increasing use of vaccination lead to the requirements of the more powerful tools to trace the contamination source and to differentiate circulating field isolates from vaccine strains, to help on better understanding of the disease epidemiology and to improve the control strategies (Whithear, 1996; Kleven, 1997).

Several molecular methods have been used for MG strains differentiation. The genotyping methods have accurate degree of reproducibility, mainly during development of consistent results and these methods were easy to perform. The improvement on the molecular biology of MG (Razin *et al*., 1998) and the availability of the MG complete genome sequence have been used to evaluate Gene-Targeted Sequencing (GTS) as a typing tool for differentiating MG strains (Papazisi *et al*., 2003). GTS analysis of multiple surface protein genes was performed to have better discriminatory power than rapid analysis. As a new approach for studying the molecular epidemiology of bacterial pathogens (Enright and Spratt, 1999), the sequencing methods have been introduced. The most important observation among MG strains are the gene size polymorphisms therefore, the DNA polymorphism is the base of application of PCR or PCR based methods for detection of MG and strains differentiation. Analysis of the gene target sequencing might help the specific strain primers synthesis and can be used for MG strains detection (Nascimento *et al*.,

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1991). The degrees of interspecies heterogeneity in these genes were evaluated as a possible strategy to differentiate between MG isolates (Liu *et al*., 2001; Ferguson *et al*., 2005). In Malaysia, the outcome of MG infection in chicken embryos with different control measures was caseous airsac when there is presence of gene size polymorphism in the *pMGA* and *pvpA* genes. The field local isolates exhibited gene size polymorphism in *pvpA* and *pMGA* genes and both genes have significant pattern of pathogenic MG field strains compared to the vaccine strain (Tan, 2008). However, there is lack of study on the molecular pathogenicity of MG infection in poultry and it is important to evaluate the molecular level of MG pathogenicity using the commercial birds and progenies (pipped embryos, normal chicks and poor quality chicks).

In this study, two Genes-Targeted Sequencing (GTS) approach to identify and differentiate MG strains was used. Therefore, the objectives of this study were carried out to evaluate the molecular pathogenic markers as a genetic biomarker for MG to determine the possible gene that can be used as a vertical transmission pathogenic marker of MG and differentiation between the high pathogenic MG isolates and less pathogenic MG isolates and to correlate the molecular findings towards existing pathogenicity of the MG strains.

MATERIALS AND METHODS

***Mycopalsma gallisepticum* reference strains and isolates:** The total of 3056 swab samples were obtained from selected commercial farms (breeder, broiler and layer), Progeny-Pipped Embryos (PE) and day old chicks [Poor Quality Chicks (PQC) and Normal Chicks (NC)] obtained from Peninsular Malaysia. The total of 571 positive samples of MG out of 3056 swab samples was detected. The samples size, the location, the molecular detection method of these 571 positive MG isolates was described by Faisal *et al.* (2011). A total of 255 positive MG out of 571 positive samples obtained from progenies samples, 316 positive MG out of 571 positive samples obtained from commercial chickens samples, reference strains (MGS6, R, PG31, A5969) and vaccine strains (6/85, TS11, F) were used in this study.

Extraction of the genomic DNA: Genomic DNA was extracted using a conventional salt based method with some modifications (OIE, 2004). A volume of 70 μL 10% SDS was added followed by adding a 1 μL of 50 μg μL^{-1} Proteinase K. The mixtures of Mycoplasma broth, SDS solution and Proteinase K were vortexed and incubated at 65°C for 30 min. The lysed samples were placed in the refrigerator at 4°C for 30 min. Precipitation of protein and peptide was achieved by adding 300 μL ammonium acetate and vortexing the mixture. The debris was pelleted by centrifugation for 10 min at 14,000 rpm. A volume of 850 μL of the supernatant was transferred and the pellet was discarded. Total nucleic acid was precipitated by adding 550 μL of isopropanol then the total nucleic acids were pelleted by centrifugation at 14,000 rpm at 4°C for 10 min. Then, the isopropanol was poured off without dislodging the nucleic acids pellet. The total nucleic acid pellet was centrifuged twice with 1 mL 75% ethanol for 10 min at 14,000 rpm. The ethanol was poured off and the nucleic acid samples were placed in the laminar air flow chamber. The DNA samples were resuspended in 50 μL of double distilled water and used for polymerase chain reaction method. The detection procedure of the 571 positive MG isolates was previous described (Faisal *et al*., 2011).

Polymerase chain reaction targeted genes: PCR procedure was performed according to the method described by Marois with some modifications. The positive samples were characterized by molecular methods targeted two genes sequence encoding of the surface protein. These two *pMGA* and *pvpA* genes have gene size polymorphism on specific target sequence (Tan, 2008). The primer sets used in this study, sequences of the primers, position and the expected amplification product size for the targeted *pMGA* and *pvpA* genes are as shown in Table 1. Optimization was carried out by varying the PCR conditions e.g., DNA concentration, amplification cycle number and primer annealing temperature. The amplifications were performed in an automatic thermal cycler (MyCycler, BioRad, USA) at 94°C for 5 min and 40 cycles of 94°C for 60 sec, 52 and 58°C for 60 sec, 72°C for 90 sec and 72°C for 5 min. The optimal annealing temperature utilized to amplify both genes are shown in Table 1. The primers were synthesized by First Base Laboratories Sdn. Bhd., Selangor. The reaction volume was set up in 25 μL reaction mixture reagents as shown in Table 2. The PCR amplified DNA fragments were detected in 1.5% agarose gel electrophoresis in TAE (1x) performed at 90v/30 min, stained with ethidium bromide (10 mg mL^{-1}) and bands visualized on UV light and the photograph was taken with analyzer (Syngene, Gene Genius Bioimaging System). The expected amplification product size ranged between 229-335 bp for *pMGA* gene and the expected amplification product size ranged between 600-702 bp for *pvpA* gene. The size of the amplified product was compared using a 100 bp plus DNA ladder (Vivantis®, Malaysia).

DNA purification, gene sequencing and data analysis: The strong positive bands on the gel were selected from both of the characterized genes and preceded to the

Table1: Product sizes and sequence positions for primers used for MG characterization

Primers	Genes	Sequence 5'-3'	PCR product size (bp)	Optimal annealing temp (°C)
AU TS11 F	*pMGA* or *vlhA* genes family (Hemagglutinin protein)	TCTTCTTCGAAAACAAAAGG	~ 329	52
AT TS11 R		GTTTGGAGTTGGTGTATAGTTAG		
pvpA 1F	Phase-variable putative adhesin protein (*pvp*A)	GCCAMTCCAACTCAACAAGCTGA	~ 702	58
pvpA 2R		GGACGTSGTCCTGGCTGGTTAGC		

Table 2: Reagents used in the PCR master mixture reaction

Reagents	Quantity
10×PCR buffer	2.5 µL (10x) (Vivantis®, Malaysia)
$MgCl_2$	2.5 µL (50 mM (Vivantis®, Malaysia))
dNTPs	1 µL (10 mM each (Vivantis®, Malaysia)
Forward primer	1 µL (25 pmole)
Reverse primer	1 µL (25 pmole)
Taq polymerase	0.2 µL (15 mM, Vivantis®, Malaysia)
DNA template	2 µL (656 ng)
Distilled water	Add to a final volume of 25 µL
Total volume per tube	25 µL

purification of the DNA. Gel purification was achieved with GENErALL PCR SV (103-1xx) purification kits. The purified DNA of the samples containing the genes of interest was sent to Macrogen Inc, Seoul, Korea for sequencing services that specialized in gene sequencing. Gene target sequencing was based on the sequence analysis of PCR amplified target sequences in each of *pMGA* and *pvpA* partial genes which encoded on the surface protein of MG. Also purified DNA of the reference strains (MGS6, R strain, PG31 strain and A5969 MG strain) and the purified DNA of the vaccine strains (ts11, 6/85 and f strain) were sent for gene sequencing. DNA sequences from different MG isolates were aligned and compared with reference and vaccine MG strains. The information gathered in the gene sequencing was compounded for the data analysis using computerized software (Bioedit software and MEGA 4).

RESULTS AND DISCUSSION

Amplification of *Mycoplasma gallisepticum* positive isolates by PCR: Out of the total samples detected positive MG isolates (571), 255 positive MG were obtained from progenies samples and 316 positive MG were obtained from commercial chickens samples as previously described by Faisal *et al.* (2011). They were preceded with molecular characterization by amplification of selected gene target specific sequences to *MG pvpA* and *pMGA* genes using conventional PCR of published sequence specific primers. These two genes, *pMGA* and *pvpA* genes have gene size polymorphism on specific target sequence. The expected amplification product size targeted *pMGA* gene ranged between 229-335 bp. The expected amplification product size targeted *pvpA* gene ranged between 600-702 bp. The PCR results with primers

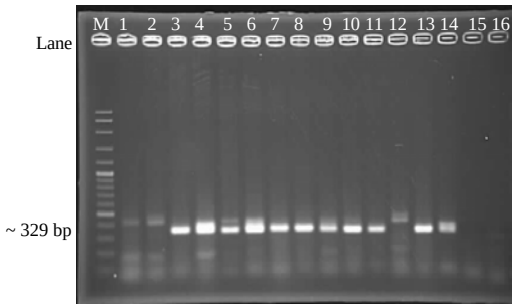

Fig. 1: PCR product of ~329 bp of MG positive isolates from progenies samples amplified using the AU-AT TS11 F+R primer set. Agarose gel electrophoresis of PCR on 16 field MG positive isolates from progenies samples. 11 MG positive isolates were positive and 5 MG positive isolates were negative by PCR with ~329 bp expected product

targeted *pMGA* gene in the progenies MG samples showed detection of a total of 169 MG positive out of 255 MG samples. Most of the progenies positive samples did not have gene size polymorphisms on the agarose gel electrophoresis but few of the samples have gene size polymorphisms (Fig. 1).

The PCR results with primers targeted *pMGA* gene in commercial chickens samples showed, a total of 112 MG positive out of 316 MG samples were detected. Most of the commercial positive samples showed clear gene size polymorphisms on the agarose gel electrophoresis (Fig. 2).

The PCR results with primers targeted for *pvpA* gene in the progenies samples, a total of 138 MG positive out of 255 MG samples were detected. Most of the progenies positive samples did not have gene size polymorphisms on the agarose gel electrophoresis but few of the samples have gene size polymorphisms (Fig. 3).

The PCR results with primers targeted for *pvpA* gene, in commercial chicken samples, a total of 50 MG positive out of 316 MG samples were detected. Most of the commercial positive samples showed clear gene size polymorphisms on the agarose gel electrophoresis (Fig. 4 and 5).

Gene targeted sequences: The amplified product size of *pMGA* and *pvpA* genes for the positive MG samples were

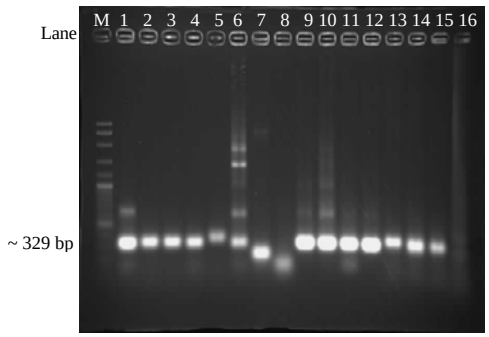

Fig. 2: PCR product of ~329 bp of MG positive isolates from commercial samples and reference strains amplified using the AU-AT TS11 F+R primer set. Agarose gel electrophoresis of PCR on 8 field MG positive isolates from commercial chickens, 8 reference and vaccine strains. The 7 MG positive isolates were positive and 1 MG positive isolates were negative by PCR with ~329 bp expected product. M = VC 100 bp plus DNA Ladder, Lane 1 = MGS6 strain, Lane 2 = PG31 strain, Lane 3 = R strain, Lane 4 = HF strain, Lane 5 = A5969, Lane 6 = F strain, Lane 7 = TS11, Lane 8 = 6 /85, Lane 9-15 = positive MG field isolates tested

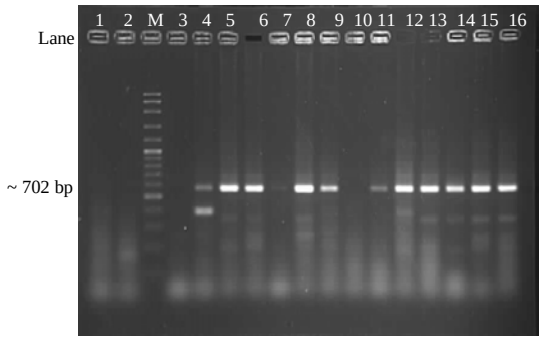

Fig. 3: PCR product of ~702 bp of MG positive isolates from progenies samples amplified using the pvpA 1F+2R primer set. Agarose gel electrophoresis of PCR on 16 field MG positive isolates from progenies. The 10 MG positive isolates were positive and 6 MG positive isolates were negative by PCR with ~702 bp expected product

analyzed and sequenced. Gene sequence of vaccine strains (ts11, 6/85 and f strain) and reference strains (MGS6 and R strain) were also analyzed and sequenced. The amplicons of the *pMGA* and *pvpA* genes were sequenced by Macrogen Inc. and nucleotide sequence editing and analysis were conducted using Bioedit software whereas alignments were conducted using MEGA4 software. Alignment of nucleotides sequence

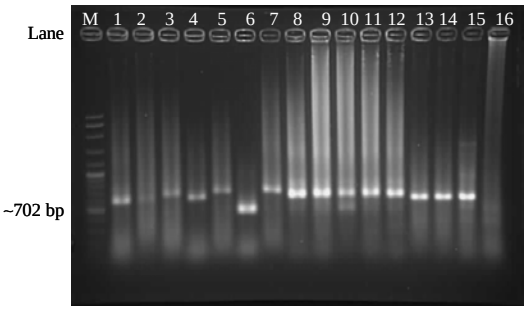

Fig. 4: PCR product of ~702 bp of MG positive isolates from commercial chicken samples, reference and strains amplified using the pvpA 1F+2R primer set. Agarose gel electrophoresis of PCR on 8 positive MG isolates from commercial chickens, 8 reference and vaccine strains. The 7 MG positive isolates were positive and 1 MG positive isolates were negative by PCR with ~702 bp expected product. M = VC100 bp plus DNA Ladder, Lane 1 = MGS6 reference strain, Lane 2 = PG31 reference strain, Lane 3 = R reference strain, Lane 4 = HF reference strain, Lane 5 = A5969 reference strain, Lane 6 = F vaccine strain, Lane 7 = TS11 vaccine strain, Lane 8 = 6/85 vaccine strain, Lane 9-15 = Positive MG isolates tested

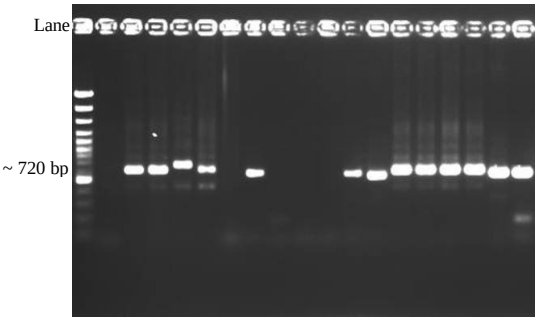

Fig. 5: PCR product of ~702 bp of MG positive isolates from commercial chicken samples amplified using the pvpA 1F+2R primer set. Agarose gel electrophoresis of PCR on 14 positive MG isolates from commercial chickens and 3 reference strains. Ten field positive isolates were positive and 4 field positive isolates were negative by PCR with ~702 bp expected product. M = VC 100 bp plus DNA Ladder, N = Control, Lane 1 = MGS6, Lane 2 = MG UK field strain, Lane 3 = ts11, Lane 4, 6, 10-17 = positive MG isolates from commercial chickens farms

analysis data demonstrated that *pMGA* gene amplification product size of all isolates have a nucleotide insertion

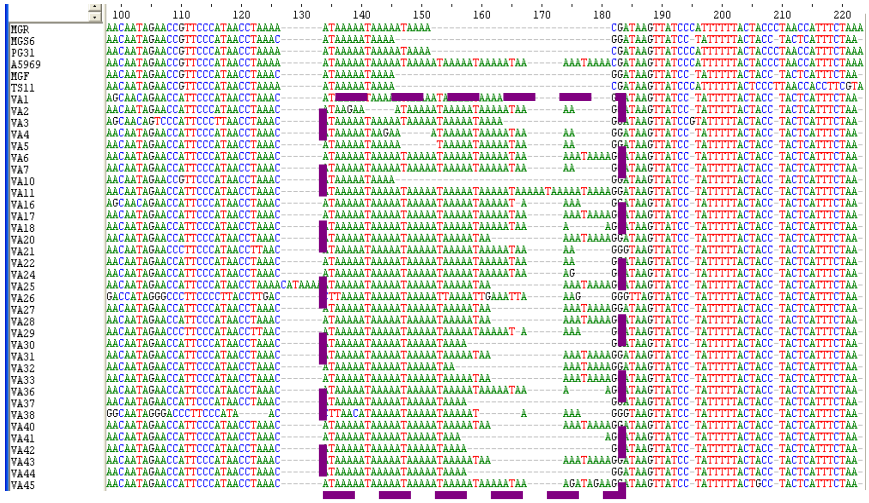

Fig. 6: Nucleotide sequences alignment of the *pMGA* gene from vaccine, reference and MG field isolates

Fig. 7: Nucleotide sequences alignment of the *pvpA* gene from vaccine, reference and MG field isolates

(Fig. 6) and the *pvpA* gene amplification product size of all isolates have a nucleotide deletion (Fig. 7) comparing with vaccine and reference strains. Gene size differences for the nucleotides sequence for *pMGA* and *pvpA* gene for each isolate, vaccine strains and reference strain were confirmed and the nucleotides sequence for them were calculated and tabulated. MG field samples isolates exhibited gene size polymorphisms in *pMGA* and *pvpA* genes with presence of insertion or deletion observed in PCR products. Gene size differences of the nucleotide target sequences of the *pMGA* gene for the MG progenies isolates comparing with high pathogenic MGS6 reference strain showed the large insertion observed was that of 35 bp fragment and comparing with the less pathogenic ts11 vaccine strain showed the largest insertion observed was that of 126 bp fragment (Table 3). However, the gene size differences of the nucleotide target sequences of the *pMGA* gene for the MG commercial chickens isolates comparing with high pathogenic MGS6 reference strain showed the large insertion observed was that of 78 bp fragment but comparing with the less pathogenic ts11 vaccine strain showed the largest insertion observed was that of 169 bp fragment (Table 4). Gene size differences of the nucleotide target sequences of the *pvpA* gene for the MG progenies isolates comparing with high pathogenic MGS6 reference strain showed the large deletion observed was hat of 38 bp fragment and comparing with the less pathogenic ts 11 vaccine strain showed the largest deletion observed was that of 97 bp fragment (Table 5). But the gene size differences of the nucleotide

Table 3: The differences of the nucleotides on the gene size of *pMGA* gene for the isolates from the progenies samples comparing with reference and vaccine strains

NO	Isolates	pMGA bp	MGS6 strain 320 bp	TS11 229 bp	NO	Isolates	pMGA bp	MGS6 strain 320 bp	TS11 229 bp
VA1	VTP3 PE 4	337	17	108	VA29	VTP27 A PE 12	345	25	116
VA2	VTP3 PE 6	338	18	109	VA30	VTP27 B PE 25	331	11	102
VA3	VTP3 PQC 1	340	20	111	VA31	VTP27 B PE 27	343	23	114
VA4	VTP5 H2 PE 10	339	19	110	VA32	VTP27 D PE 8	337	17	108
VA5	VTP5 H2 PE 13	337	17	108	VA33	VTP27 D PE18	344	24	115
VA6	VTP7 14WK PE 4	351	31	122	VA36	VTP27 A PQC 5	343	23	114
VA7	VTP 7 55WK PE 10	343	23	114	VA37	VTP28 B PE 2	331	11	102
VA10	VTP9 H4 PQC 1	319	-1	90	VA38	VTP28 B PE 5	335	15	106
VA11	VTP10 FA PE 7	335	15	106	VA40	VTP28 E PE 10	343	23	114
VA16	VTP14 DC 3	343	23	114	VA41	VTP28 A PQC 6	331	11	102
VA17	VTP14 DC 6	349	29	120	VA42	VTP28 B PQC 1	331	11	102
VA18	VTP14 PE 3	343	23	114	VA43	VTP28 B PQC 2	343	23	114
VA20	VTP19 R4 PE 5	343	23	114	VA44	VTP28 C PQC 5	331	11	102
VA21	VTP19 R6 PE 6	345	25	116	VA45	A17 PQC 1	349	29	120
VA22	VTP19 R7 PE 1	343	23	114	VA46	A17 PE 1	325	5	96
VA24	VTP20 50WK PE 7	347	27	118	VA47	A17 PE 2	355	35	126
VA25	VTP20 50WK PE 10	351	31	122	VA48	A21 PC 3	355	35	126
VA26	VTP20 60WK PE 20	355	35	126	VA50	A21 PE 8	355	35	126
VA27	VTP20 60WK PE 25	343	23	114	VA51	A18 PE 4	355	35	126
VA28	VTP27 A PE 11	343	23	114	VA52	VTP1 H6 PE 1	317	-3	88

Table 4: The differences of the nucleotides on the gene size of *pMGA* gene for the isolates from the commercial samples comparing with reference and vaccine strains

No	Isolates	pMGA bp	MGS6 strain 320 bp	TS11 229 bp	No	Isolates	pMGA bp	MGS6 strain 320 bp	TS11 229 bp
CA3	HF	323	3	94	CA25	C 58	368	48	139
CA5	PF3U	350	30	121	CA26	D 2	367	47	138
CA6	PF7U	331	6	102	CA27	D7	372	52	143
CA7	3B	331	11	102	CA29	F 21	324	4	95
CA8	W7A 4	330	10	101	CA30	ZAH 2	338	18	109
CA10	W7B 8	398	78	169	CA31	AK9	343	23	114
CA11	KUN 20 WK 36a	332	12	103	CA32	T25	332	12	103
CA12	VL4 30 WK 3	343	23	114	CA33	EES	332	12	103
CA13	VL4 30WK 22	332	12	103	CA34	T 4	341	21	112
CA14	VL18 25WK 17	343	23	114	CA35	T 5	357	37	128
CA15	VL18 25 WK 31	327	7	98	CA36	T 6	359	39	130
CA16	SHL1 5C	337	17	108	CA37	T 7	350	30	121
CA17	SHL2 3as	354	34	125	CA38	T 8	355	35	126
CA18	KUN20WK 51	338	18	109	CA39	T 9	354	34	125
CA19	KUN20WK 23a	332	12	103	CA40	T 10	349	29	120
CA20	A 70	361	41	132	CA41	T 11	356	36	127
CA22	B 29	319	-1	90	CA42	T 12	356	36	127
CA23	B 37	336	16	107	CA43	T 13	356	36	127
CA24	B 54	375	55	146	-	-	-	-	-

Table 5: The differences of the nucleotides on the gene size of *pvpA* gene for the isolates from the progenies samples comparing with reference and vaccine strains

No	Isolates	pvpA bp	MGS6 610 bp	TS11 669 bp	No	Isolates	pvpA bp	MGS6 610 bp	TS11 669 bp
Vp1	VTP3 PE 4	572	-38	-97	VP23	VTP20 50WK PE 10	572	-38	-97
Vp2	VTP3 PE 6	572	-38	-97	VP24	VTP20 50WK PE 15	572	-38	-97
VP3	VTP3 PQC 1	572	-38	-97	VP25	VTP20 60WK PE 20	572	-38	-97
VP4	VTP5 H2 PE 10	572	-38	-97	VP26	VTP20 60WK PE 25	572	-38	-97
VP5	VTP5 H2 PE 13	572	-38	-97	VP28	VTP27 A PE 2	572	-38	-97
VP6	VTP7 14WK PE 4	572	-38	-97	VP29	VTP27 A PE 14	572	-38	-97
VP7	VTP7 55WK PE 20	572	-38	-97	VP30	VTP27 B PE 1	572	-38	-97
VP8	VTP7 55WK PE 21	572	-38	-97	VP31	VTP27 D PE 22	572	-38	-97
VP9	VTP7 55WK PE 29	572	-38	-97	VP32	VTP27 E PE 1	573	-37	-96
VP10	VTP10 FA PE 7	573	-37	-96	VP33	VTP27 E PE 14	573	-37	-96
VP11	VTP10 FA PE 12	572	-38	-97	VP34	VTP27 A PQC 4	573	-37	-96
VP13	VTP14 NC 14	572	-38	-97	VP35	VTP27 A PQC 5	572	-38	-97
VP14	VTP14 DC 6	572	-38	-97	VP36	VTP28 E PE 6	572	-38	-97
VP15	VTP 14 DC 9	573	-37	-96	VP37	VTP28 E PE 10	574	-36	-95
VP16	VTP14 PE 14	572	-38	-97	VP38	VTP28 B PQC 10	572	-38	-97
VP17	VTP14 PE 16	572	-38	-97	VP39	VTP28 B PQC 12	572	-38	-97
VP19	VTP19 R6 PE 8	572	-38	-97	VP40	A17 PE 1	574	-36	-95
VP20	VTP19 R7 PE 1	572	-38	-97	VP42	A21 PE 2	574	-36	-95
VP21	VTP19 R7 PE 12	572	-38	-97	VP43	A18 PE 4	572	-38	-97
VP22	VTP20 50WK PE 7	572	-38	-97	VP44	VTP1 H6 PE 1	572	-38	-97

sequences of the *pvpA* gene for the MG commercial chickens isolates comparing with high pathogenic MGS6 reference strain showed the large deletion observed was that of 43 bp fragment and comparing with the less pathogenic ts11 vaccine strain which showed the largest deletion observed was that of 102 bp fragment (Table 6). In this study, the PCR results with primers targeted for *pMGA* and *pvpA* genes showed that most of the vertically transmitted positive samples were absent or had slightly gene size polymorphisms on the agarose gel electrophoresis. It was confirmed by nucleotides sequence analysis with approximately less of gene size polymorphisms. In contrast, most of the commercial positive isolates showed clear gene size polymorphisms on the agarose gel electrophoresis which was confirmed by nucleotides sequence analysis. The results support previous observation reported that all the MG house finch isolates using RAPD banding patterns method had identical RAPD patterns and showed a single, unique profile, distinct from those of chicken or turkey isolates tested suggesting a single source of MG in house finches (Hartup *et al*., 2000; Liu *et al*., 2001).

The results of this study showed that the commercial chicken isolates yielded gene size between 319-398 bp PCR products with the pMGA (AU-AT TS11) primer as shown in Table 4, the progenies isolates yielded gene size between 317-355 bp (Table 3) while gene size between 229-353 bp was shown for vaccine and reference strains (Table 7). All the positive isolates were tested for the presence of *pMGA* gene using a proprietary PCR primer that was specifically designed to identify the ts11 strain. This assay amplified a 229 bp fragment that was characterized of ts11 less pathogenic vaccine strain. All other MG field strain isolates that were amplified with this assay produced a different sized patterns and can therefore, be distinguished from the ts11 vaccine strain but only the MG 6/85 vaccine strain cannot be amplified by the PMGA (AU-AT TS11) primer. Although, these data are different from other study which showed that there was one field strain isolated from the USA has been found to amplify with similar gene size to that of ts11 and the AUAT ts11 specific primer assay amplified the 6/85 vaccine but the two strains were easily distinguishable from each other since the gene size were different among MG strains (Kleven, 2002). Another study used a designed ts11 specific primer sets for strains differentiation by the conventional PCR and the differentiation was based on the similar bands size of the amplification product on the gel for the less pathogenic vaccine strains ts11 and 6/85. Lack of the amplification products on the gel was observed from the pathogenic MG R, R low and MGS6 strains. It can also differentiate MG ts11 and MG 6/85 strains from all field MG strains including those sharing a high degree of genetic similarity (Evans and Leigh, 2008).

The commercial chicken isolates yielded gene size polymorphisms between 567-611 bp PCR products with the pvpA primer as shown in Table 6. The progenies isolates yielded gene size 572-574 bp (Table 5) while gene size between 439-669 bp was observed for vaccine and reference strains (Table 7). However, previous studies (Boguslavsky *et al*., 2000; Liu *et al*., 2001; Pillai *et al*., 2003), reported that the *pvpA* gene exhibited size polymorphisms with PCR products of 437, 578, 606 and 665 bp as detected among MG reference strains and isolates. The region of the *pvpA* gene amplified encodes the protein carboxy-terminus where truncations of the pvpA protein have been reported to be located within the proline-rich Direct Repeat (DR) (Boguslavsky *et al*., 2000; Ferguson *et al*., 2005). In the present study, only the vaccine MGF strain showed the largest deletion observed and it was considered specific *pvpA* gene size 439 bp which is different from other vaccine, reference and circulating field isolates. These results suggested that the MG-F vaccine strain showed molecular gene size marker that can be differentiated from other MG strains. Another research also showed the largest deletion observed was that of a 230 bp fragment in vaccine strain F (Liu *et al*.,

Table 6: The differences of the nucleotides on the gene size of *pvpA* gene for the isolates from the commercial samples comparing with reference and vaccine strains

NO	Isolates	pvpA bp	MGS6 610 bp	TS11 669 bp
CP6	3B	572	-38	-97
CP10	VL4 30WK 22	575	-35	-94
CP12	SHL1 5C	567	-43	-102
CP13	KUN 30WK 51	576	-34	-93
CP14	KUN 30WK 23A	572	-38	-97
CP15	D 49	567	-43	-102
CP16	D 56	567	-43	-102
CP17	ZAH 2	611	1	-58
CP18	AK9	574	-36	-95

Table 7: The difference in nucleotides sequences of the *pMGA* and *pvpA* genes among vaccine and reference strains

Reference and vaccine strains	Type	pMGA (bp)	pvpA (bp)
MGS6 strain	Pathogenic reference strain	320	610
R strain	Pathogenic reference strain	328	665
PG31 strain	Pathogenic reference strain	327	611
A5969 strain	Pathogenic reference strain	353	666
F strain	Pathogenic vaccine strain	321	439
6/85 strain	Mild vaccine strain	Not amplified	608
Ts11 strain	Mild vaccine strain	229	669

target 2001; Jiang *et al*., 2009). Previous sequence analysis of MG reference strain A5969 demonstrated that this strain possesses a full length of *pvpA* structural gene, similar in size to the *pvpA* gene from strain R. Both of these strains have a complete DR sequences and yield a 665 bp PCR product (Boguslavsky *et al*., 2000; Ferguson *et al*., 2005; Jiang *et al*., 2009) which was similar to the data obtained in this study where the *pvpA* gene nucleotides sequence from MG R strain yield a product size 665 bp and *pvpA* gene nucleotides sequence from MG A5969 strain yield a product size 666 bp.

Sequence analysis for the MG field isolates in this study showed that in addition to nucleotide sequence variability, gene size polymorphisms of the *pvpA* and *pMGA* genes was observed among MG strains and this finding is similar to the previous study obtained by Boguslavsky *et al*. (2000), Liu *et al*. (2001), Pillai *et al*. (2003), Papazisi *et al*. (2003), Evans *et al*. (2005) and Jiang *et al*. (2009). Nucleotides sequence analysis data demonstrated that the *pMGA* gene amplification product size of all isolates have nucleotides insertion as shown in the alignments (Fig. 6) and *pvpA* gene amplification product size of all isolates have a nucleotide deletion as shown in the alignments (Fig. 7), comparing with high pathogenic reference strain MGS6 and less pathogenic vaccine strain ts11. This is considered a specific gene size marker to the MG local field isolates and is different from isolates in other places. The sequence analysis confirmed that the MG field isolates exhibited gene size polymorphisms in *pMGA* and *pvpA* genes with presence of insertion or deletion observed in PCR products. These findings are similar to those obtained by Jaganathan (2007) and Tan (2008). It also confirmed that both genes have significant gene size pattern of pathogenic MG field strain compared to the vaccine strain. This is similar to the study reported by Tan (2008). The results support previous observation on the sequence analysis within the *pvpA* gene that showed the molecular variation patterns of the *pvpA* genes among 15 MG field isolates were similar to the pathogenic reference strain S6 and BG44T that is a 60 bp deletion in DR-1 and DR-2. However, the molecular variation pattern is quite different from that of the vaccine strain F36, size variation and variation in the patterns of the deletion region of the *pvpA* gene observed in the F vaccine strain and among the field MG isolates that help on the differentiation among MG strains (Jiang *et al*., 2009).

MG strains differ in their potential for producing embryo mortality and most of the strains are pathogenic for chicken embryos (Yoder and Hofstad, 1964). Previous pathogenicity experiment showed that the MG field isolates MI-211 caused higher embryo mortality in the early period of incubation (3-5 days post inoculation). This was comparable with the embryo mortality pattern shown by pathogenic reference strain MGS6 (Jordan, 1979). On the other hand, the three strains MI-200, MI-203 and MI-225 were found to be similar in their embryo mortality pattern as all of them caused embryo mortality in the late phase of incubation (5-10 days post inoculation). This pattern was comparable with the embryo mortality pattern shown by the vaccine strain MGF (Cummings and Kleven, 1986; Glisson and Kleven, 1984). Pathogenicity study in Malaysia showed that based on the presence of the gene size polymorphisms in *pvpA* and *pMGA* genes, I44 and I-18 (local MG strains) have a similar pattern of pathogenicity with the MGS6 (reference strain), in that they are highly pathogenic and caused early embryonic death. While H21 8T, H21 11T, H24 5C and H26 9C (local MG strains) have similar pattern of pathogenicity with the vaccine strain ts-11, in that they are less pathogenic in embryos and caused embryo mortality during later stages of incubation (Tan, 2008).

The correlation between the molecular findings in this study with the previous pathogenicity studies revealed that when the (AU-AT ts11) primer of the *pMGA* gene size nucleotides sequence of the positive isolates was on the expected size ~329 bp or between 300-350 bp, consequently means that the positive isolate is pathogenic and able to transmit vertically, like the pattern of the pathogenic MGS6, F strain and R strains. On the other hand when the gene size nucleotides sequence of the positive isolates located between 200-250 bp which suit to the *pMGA* gene size of MG ts11 229 bp (less pathogenic MG vaccine strain) consequently means that these isolates are less pathogenic and cannot be transmitted vertically because ts11 vaccine strain was mild or less pathogenic. From these data the reachers can postulate that this partial nucleotides sequence of *pMGA* gene can be used as a MG vertical transmission molecular marker. The results of primer targeted *pvpA* gene showed the band produced was different between isolates and the gene size was between 600-702 bp. The positive isolates, approximately 669 bp which fit to the *pvpA* gene size of the ts11 or above, consequently indicated that these isolates were less pathogenic or mild isolates. However, when the positive isolates were <669 bp or range between 550-669 bp which was similar to the range pattern of the pathogenic MG reference strains and to the MG field isolates in the present study, accordingly indicated that these isolates were pathogenic. Another interesting finding related to MG of mild vaccine strain 6/85 was that no expression of the pMGA product obtained was from the whole population of strain 6/85. However, this strain possesses a *pvpA* gene expression approximately similar

in size to its counterpart in MGS6 which was pathogenic. Therefore, the researchers hypothesized from this finding that when the positive isolates was not amplified by the pMGA and it was amplified using the *pvpA* gene. These positive isolates are considered as mild pathogenic MG strain because these isolates on the molecular level behave like the mild vaccine strain 6/85 which was not amplified by pMGA. This might be because MG 6/85 did not have this partial gene itself or it does not have the nucleotides sequence of the target primer while 6/85 can amplify using *pvpA* gene. Since, pMGA was apparently an adhesin-related surface molecule of MG, it was interesting to speculate whether lack of this partial nucleotides sequence of pMGA expression contributes in part to the deficiency in the adherence capabilities and virulence of vaccine strain MG 6/85.

CONCLUSION

The general conclusion of the results in the current study indicated that while there might be considerable genotypic homology (identical patterns) among MG isolates from progenies, more diversity patterns were observed in this study among the isolates from commercial chickens. The molecular variation of the *pMGA* and *pvpA* genes among MG field isolates shared similar genetic variations patterns with the pathogenic reference and vaccine strains that is an insertion bp fragments by using the *pMGA* gene primer set and a deletion of bp fragments by using the *pvpA* gene primer set. The MG field isolates have identical genes size variation patterns with the pathogenic vaccine and reference strains which are pathogenic by nature and can be transmitted vertically. Therefore, this study confirmed that using *pMGA* gene as a pathogenic vertical marker and the combination of the gene size polymorphisms patterns in both selected primers of *pMGA* and *pvpA* genes are considered as two genetic potential pathogenic markers. This is because the primers sites used in this study were different in different MG strains. Therefore, these primers sites might be useful in distinguishing between the pathogenic and less pathogenic MG strain and it looks likes these primers sites are related to virulence factors of MG.

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