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Use of molecular diversity of *Mycoplasma gallisepticum* by gene-targeted sequencing (GTS) and random amplified polymorphic DNA (RAPD) analysis for epidemiological studies

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A total of 67 *Mycoplasma gallisepticum* field isolates from the USA, Israel and Australia, and 10 reference strains, were characterized by gene-targeted sequencing (GTS) analysis of portions of the putative cytadhesin *pvpA* gene, the cytadhesin *gapA* gene, the cytadhesin *mgc2* gene, and an uncharacterized hypothetical surface lipoprotein-encoding gene designated genome coding DNA sequence (CDS) MGA_0319. The regions of the surface-protein-encoding genes targeted in this analysis were found to be stable within a strain, after sequencing different *in vitro* passages of *M. gallisepticum* reference strains. Gene sequences were first analysed on the basis of gene size polymorphism. The *pvpA* and *mgc2* genes are characterized by the presence of different nucleotide insertions/deletions. However, differentiation of isolates based solely on *pvpA*/*mgc2* PCR size polymorphism was not found to be a reliable method to differentiate among *M. gallisepticum* isolates. On the other hand, GTS analysis based on the nucleotide sequence identities of individual and multiple genes correlated with epidemiologically linked isolates and with random amplified polymorphic DNA (RAPD) analysis. GTS analysis of individual genes, *gapA*, MGA_0319, *mgc2* and *pvpA*, identified 17, 16, 20 and 22 sequence types, respectively. GTS analysis using multiple gene sequences *mgc2*/*pvpa* and *gapA*/MGA_0319/*mgc2*/*pvpA* identified 38 and 40 sequence types, respectively. GTS of multiple surface-protein-encoding genes showed better discriminatory power than RAPD analysis, which identified 36 pattern types from the same panel of *M. gallisepticum* strains. These results are believed to provide the first evidence that typing of *M. gallisepticum* isolates by GTS analysis of surface-protein genes is a sensitive and reproducible typing method and will allow rapid global comparisons between laboratories.

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INTRODUCTION

Mycoplasma gallisepticum is a major problem in the poultry industry worldwide, causing chronic respiratory disease of chickens and turkeys. Control of *M. gallisepticum* has generally been based on eradication of the organism from breeder flocks and maintenance of mycoplasma-free status in the breeders and their progeny by implementation of biosecurity. The rapid and widespread expansion of poultry in restricted geographical areas and the consequent re-emergence of mycoplasma infection have necessitated a re-evaluation of the strategies utilized to control mycoplasma infections of poultry. In areas where complete eradication is difficult to attain, vaccination with live vaccines is utilized as an alternative control strategy (Whithear, 1996; Kleven, 1997). Consequently, with the increased use of vaccination, more powerful tools to trace the source of contamination and to differentiate vaccine strains from circulating field

Abbreviations: CDS, coding DNA sequence; DR, direct repeat; GTS, gene-targeted sequencing; p, passage; RAPD, random amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this work are AY556071–AY556382.

Six dendrograms constructed for GTS analysis are available as supplementary data with the online version of this paper.

isolates are required to aid better understanding of the epidemiology of the disease and to improve control strategies.

Several techniques have been developed for differentiation of *M. gallisepticum* strains, including protein profile analysis (Khan *et al.*, 1987), restriction fragment length polymorphism (RFLP) (Kleven *et al.*, 1988), ribotyping (Yogev *et al.*, 1988), strain-specific DNA probes (Khan *et al.*, 1989) and PCR with strain-specific primers (Nascimento *et al.*, 1993). However, none of these methods has been as widely used as random amplified polymorphic DNA (RAPD) (Charlton *et al.*, 1999b; Fan *et al.*, 1995; Geary *et al.*, 1994). The RAPD method has been successfully utilized to identify vaccine strains in both experimental and field conditions (Ley *et al.*, 1997b; Kleven & Fan, 1998; Turner & Kleven 1998), as well as for tracking epidemiologically related isolates in the field (Kempf, 1998; Ley *et al.*, 1997a; Charlton *et al.*, 1999a; Levisohn & Kleven, 2000). However, the use of the RAPD method has not allowed for inter-laboratory comparisons or long-term epidemiological studies due to difficulty in standardizing and unifying protocols among laboratories. The RAPD technique has intrinsic problems of reproducibility because numerous experimental parameters, such as $MgCl_2$ concentration, *Taq* polymerase concentration and source, template DNA concentration, and thermocycler program and model, all affect the reproducibility of the technique in different laboratories and over time in the same laboratory (Tyler *et al.*, 1997). Recently sequencing methods have been introduced as a new approach for studying the molecular epidemiology of bacterial pathogens (Enright & Spratt, 1999). Multilocus sequence typing of housekeeping genes has been demonstrated to be a highly transferable typing method, readily applicable to a wide variety of bacteria, which has contributed to the understanding of global epidemiology and population structure of infectious diseases (Maiden *et al.*, 1998).

In this study we present what is believed to be the first attempt to use a gene-targeted sequencing (GTS) approach to identify and differentiate among *M. gallisepticum* strains. Four genes were chosen for GTS analysis: three genes encoding previously characterized *M. gallisepticum* surface proteins and one gene encoding a predicted *M. gallisepticum* surface protein. The four gene sequences were identified in the genome of *M. gallisepticum* R_{low} strain (Papazisi *et al.*, 2003). The *gapA* gene encodes a protein shown to be involved in the cytadhesion process (Goh *et al.*, 1998) identified as genome coding DNA sequence (CDS) MGA_0934. The *mgc2* gene encodes a second cytadhesin protein also known to play a role in the attachment process (Hnatow *et al.*, 1998) identified as genome CDS MGA_0932. The *pvpA* gene encodes a putative accessory cytadhesin that exhibits size variation among *M. gallisepticum* strains (Boguslavsky *et al.*, 2000; Liu *et al.*, 2001) and was identified as an interrupted CDS in the *M. gallisepticum* R_{low} genome sequence by Papazisi *et al.* (2003). A 37 nt internal duplication within the *pvpA* gene resulted in a reading frame shift predicting two coding sequence fragments identified as genome CDSs MGAL_0256 and MGAL_0258, encoding the C-terminal and N-terminal ends of the *pvpA* gene products, respectively (Papazisi *et al.*, 2003). The gene encoding a predicted conserved surface lipoprotein, originally recognized by Nascimento *et al.* (1991), was identified as genome CDS MGA_0319 (Papazisi *et al.*, 2003).

Gene-targeted sequencing analysis of *M. gallisepticum* was conducted to: (1) evaluate the reproducibility and discriminatory capability of surface-protein genes as a tool for typing *M. gallisepticum* isolates; (2) compare the discriminatory power of GTS versus RAPD typing method; and (3) establish relationships among *M. gallisepticum* isolates from different outbreaks and geographical areas.

METHODS

***M. gallisepticum* isolates.** Sixty-seven *M. gallisepticum* isolates were analysed: fifty-four from the USA, five from Australia and eight from Israel. The isolates from the USA were obtained from the depository at the Poultry Diagnostic and Research Center (PDRC), University of Georgia. These isolates were from 16 different states, isolated from 1973 to 2001. They were from broiler breeders, commercial layers, turkey breeders, meat-type turkeys, house finches and an American goldfinch. The Israeli isolates were obtained from the depository of the Division of Avian and Aquatic Diseases, Kimron Veterinary Institute (Beit Dagan, Israel). These strains were isolated from *M. gallisepticum* outbreaks in broiler breeders, turkey breeders and meat-type turkeys. The outbreaks had no known epidemiological link, as was supported by initial RAPD typing performed in Israel. The Australian isolates were acquired courtesy of K. G. Whithear, from the University of Melbourne (Victoria, Australia) and included strain AU083CK80, the pathogenic field isolate from which the ts-11 vaccine was derived by mutagenesis (Soeripto *et al.*, 1989).

Reference *M. gallisepticum* strains used in this study included: 6/85 laboratory strain (29 p) (Evans & Hafez, 1992) and 6/85 vaccine (Intervet Inc., Millsboro, DE); ts-11 laboratory strain (99 p) (Whithear *et al.*, 1990) and ts-11 vaccine (Merial, Gainesville, GA); R (13, 73 and 154 p) (Rodriguez & Kleven, 1980; Levisohn *et al.*, 1986); S6 (20, 106 and 259 p) (Zander, 1961; Levisohn *et al.*, 1986); HF-51 (91 p) (Luttrell *et al.*, 1996); K503 (66 p), K703 (38 p), K730 (477 p) (Yoder, 1986); A5969 (192 p) (Van Roekel & Olesiuk 1952); F laboratory strain (16, 124 and 255 p) (Adler *et al.*, 1960; Luginbuhl *et al.*, 1967; Levisohn *et al.*, 1986) and F vaccine (Schering Plough Animal Health, Millsboro, DE). The passage levels of A5969 and F laboratory strains are recorded from the time of deposit at the PDRC depository.

PCR amplification and sequencing of targeted genes. Nucleic acid was extracted from 150 to 250 µl of a culture grown in modified Frey's medium or frozen Frey's medium stock cultures stored with 5 % (v/v) glycerol. Genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN), following the manufacturer's recommendations. Primer sequences, location and expected amplification product size for the targeted genes, *gapA*, MGA_0319 *mgc2* and *pvpA*, are shown in Table 1. Primer positions given are based on the *M. gallisepticum* R_{low} genome sequence (AE015450). All amplifications were performed in a PTC-200 DNA Engine MJ thermocycler (MJ Research) at 94 °C for 3 min, and 40 cycles of 94 °C for 20 s, 55 to 60 °C for 40 s, 72 °C for 60 s, and 72 °C for 5 min. The optimal annealing temperature utilized to amplify the MGA_0319 and *pvpA* genes was 55 °C, to amplify the *mgc2* gene an annealing temperature of 58 °C was utilized, and 60 °C was utilized to amplify

Table 1. Location, product sizes and sequence positions for primers used in GTS analysis

Primer	Sequence (5′→3′)	Location		PCR product size	GTS analysis sequence‡
		Genome CDS	nt position†		
lp 1F	CCAGGCATTTAAAAATCCCAAAGACC	MGA_0319 C*	906–931	590	955–1449
lp 1R	GGATCCCATCTCGACCACGAGAAAA		1471–1495		
gapA 3F	TTCTAGCGCTTTAGCCCTAAACCC	MGA_0934	2601–2624	332	2623–2931
gapA 4R	CTTGTGGAACAGCAACGTATTCGC		2909–2932		
pvpA 3F	GCCAMTCCAACTCAACAAGCTGA	MGAL_0258_0256 C*	545–567	702§	750–1205
pvpA 4R	GGACGTSGTCCTGGCTGGTTAGC		1224–1246		
mgc2 1F	GCTTTGTGTTCTCGGGTGCTA	MGA_0932	53–73	824	196–780
mgc2 1R	CGGTGGAAAACCAGCTCTTG		857–876		

*C, complement of the *M. gallisepticum* R_{low} genome CDS.
†Nucleotide positions of primers based on *M. gallisepticum* R_{low} genome CDS.
‡Nucleotide positions of gene fragments used in GTS analysis based on *M. gallisepticum* R_{low} genome CDS.
§Estimated PCR size includes the 37 nt internal duplication of the predicted *pvpA* MGAL_0258_0256 CDSs.

the *gapA* gene. PCR products were detected with UV light in a 2 % agarose gel containing 1 μg ethidium bromide ml^{-1}. The amplified gene fragment was sequenced using an Applied Biosystems Prism 377 automated sequencer (PE Applied Biosystems). Each amplification product was sequenced in both directions with the forward and reverse amplification primers (Table 1). Complete overlapping of complementary sequences, editing and consensus construction was performed using the SEQMAN program (in LASERGENE; DNASTAR). Sequencing of all amplification products was performed at the Integrated Biotechnology Laboratories located at the University of Georgia. Sequencing of reference strains and Israeli isolates was also carried out at the Sequencing Unit, Weizmann Institute (Rehovot, Israel).

Sequence stability of targeted genes. In order to evaluate the stability of the four targeted gene regions, amplification and sequencing was performed for several *in vitro* passages of reference strains R and S6, vaccine strains F, ts-11 and 6/85, and vaccine strains as obtained from the manufacturers.

RAPD analysis. The primers used in RAPD analysis were described by Fan *et al.* (1995). The RAPD reaction conditions were performed as described by Ley *et al.* (1997a). RAPD pattern analysis was performed by visual comparison using digitally recorded photographs of ethidium bromide stained agarose gel. All RAPD analyses were performed at PDRC University of Georgia. Each RAPD analysis gel was standardized by comparison of the *M. gallisepticum* unknown isolate to reference strains. Isolates were considered identical when major band differences could not be visualized. RAPD patterns for reference strains and isolates were designated with letters of the alphabet A to Z followed by double letter designations as new patterns appeared during the analysis. RAPD types were not designated with letters F, R and S to avoid confusion with *M. gallisepticum* reference strains F, R and S6.

GTS analysis. To conduct GTS analysis all consensus sequences were edited to start at an equivalent coding sequence position using the EDITSEQ program (in LASERGENE; DNASTAR). Nucleotide positions of analysed gene fragments are given in Table 1 based on the genome sequence of *M. gallisepticum* R_{low} strain. Alignments of individual gene and multiple gene sequences were constructed by the CLUSTAL V method with a gap penalty of 10 using the MEGALIGN program (in LASERGENE; DNASTAR). Dendrograms were constructed from the CLUSTAL V alignments by the neighbour joining method and 1000 bootstrap replicate analysis using the MOLECULAR EVOLUTIONARY GENETIC ANALYSIS (MEGA) software available at www.megasoftware.net for individual genes and for multiple genes sequence alignments. Distinct groups of closely related sequences were identified as clusters in the dendrogram and designated with roman numbers (I, II, III). Sequences within a cluster with <100 % nucleotide identity were identified as different sequence types by lower-case letters (a, b, c). For GTS analysis of individual and multiple genes each isolate received a sequence type designation indicating the cluster and sequence type they belong to. Because sequences from the same isolate did not segregate into the same cluster in each individual and multiple gene analysis, the same isolate did not necessarily receive the same cluster designation. The total number of sequence types identified by GTS analysis of individual and multiple genes was estimated and isolates were grouped based on RAPD and GTS types.

Sequences of 67 isolates and 10 reference strains were submitted to GenBank under the following accession numbers: MGA_0319, AY556071–AY556148; *gapA*, AY556149–AY556226; *mgc2*, AY556227–AY556304; *pvpA*, AY556305–AY556382.

Discrimination index of gene size polymorphism, GTS and RAPD analysis. The overall discriminatory power of a typing method is defined as its ability to distinguish between different strains. This can be expressed as an index that measures the probability that two unrelated strains will be placed into different groups, and is calculated using Simpson's diversity or discrimination (*D*) index, which takes into account the number of types defined by the method and the relative frequencies of these types (Hunter & Gaston, 1988). A *D* index of >0·90 is considered adequate, and a *D* index >0·95 is considered as a good typing discrimination power. For each gene fragment, isolates with identical sequences (100 % identity) were considered as the same sequence type. Discrimination indices were estimated for RAPD and GTS analysis of individual and multiple genes, and for gene size polymorphism of *pvpA* and *mgc2* genes.

RESULTS

Sequence stability of targeted genes

Targeted gene stability was evaluated by sequencing several *in vitro* passage levels of reference strains R and S6, and

vaccine strains F, ts-11 and 6/85. Sequences of reference strain R passaged 13, 73 and 154 times, were 100 % identical for all targeted genes. Likewise, sequences of each of the targeted genes in strain S6, at passage levels 20, 106, 259, were 100 % identical as were sequences in vaccine strain ts-11 passaged 99 times and commercial vaccine 6/85 passaged 29 times. The MGA_0319, *mgc2* and *pvpA* gene sequences of F strain, passaged 16, 124 and 255 times, and the commercial vaccine, were 100 % identical. However, two nucleotide differences (99·4 % identity) were found in the *gapA* gene of F strain passaged 124 times as compared to F strain passaged 16 and 255 times, and the commercial F strain vaccine. Nucleotide changes of F strain 124 p were located at nucleotide positions 2920 (G→C) and 2928 (A→C) of the *gapA* genome CDS MGA_0934.

RAPD analysis

A total of 77 isolates of *M. gallisepticum*, including field isolates and reference strains, were typed by RAPD analysis. As previously reported by Fan *et al.* (1995) and Ley *et al.* (1997a, b), *M. gallisepticum* reference strains, K503CK74 (RAPD type G), K703CK75 (RAPD type H), K730CK75 (RAPD type I) (Fig. 1a, lanes 1 to 3), 6/85 (RAPD type M), HF-51 (RAPD type N), ts-11 (RAPD type L), F (RAPD type B) and R (RAPD type C) (Fig. 1a, lanes 8 to 12), were readily differentiated using Fan primers. RAPD analysis of *M. gallisepticum* isolates from the USA, Australia and Israel were also typed using the Fan primer set. Eighteen different RAPD types were observed for the fifty-four USA isolates analysed. These were designated RAPD types B, E, J–Q, T–Z and AA. Among the fifty-four USA isolates, three were identified as RAPD type B, characteristic of vaccine strain F; seven were RAPD type M, characteristic of vaccine strain 6/85; and seven were RAPD type L, characteristic of vaccine strain ts-11. The remaining 37 isolates all presented RAPD patterns different from the *M. gallisepticum* reference strains. Fig. 1(a) shows an example of *M. gallisepticum* isolates characterized as RAPD types K, W, E and X (lanes 4 to 7), and Fig. 1(b) shows an example of *M. gallisepticum* isolates characterized as RAPD type O (lanes 16 to 20) and RAPD type Z (lanes 21 to 23). All eight Israeli isolates showed different RAPD types, designated AF to AM. Of the five Australian isolates, four showed unique RAPD types designated AB to AE while strain AU083CK80, the parent strain of ts-11, typed similar to the vaccine strain (RAPD type L). Results of the RAPD typing for the 77 *M. gallisepticum* isolates are presented in Table 2.

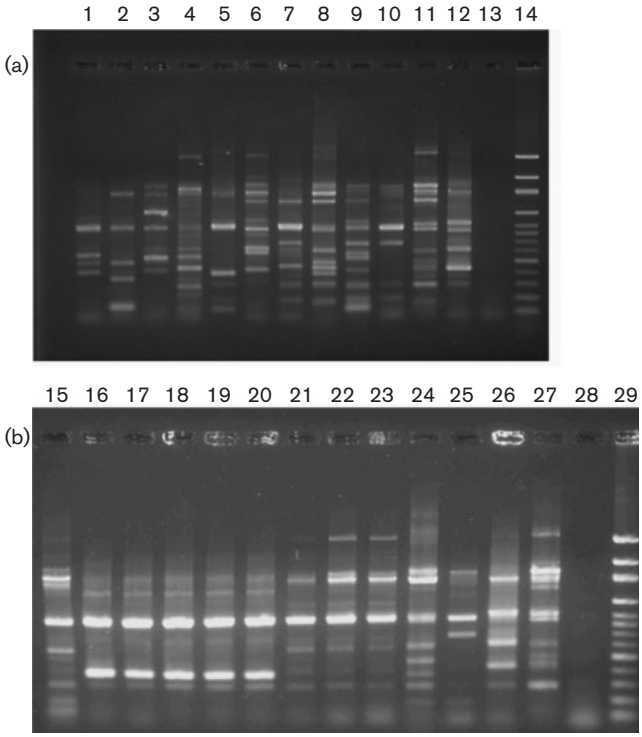

Fig. 1. RAPD analysis of *M. gallisepticum* reference strains and field isolates with the primers described by Fan *et al.* (1995). (a) Lanes 1 to 3, reference strains K503, K703, K730; lanes 4 to 7, isolates K3020TK90, K4705CK99, K435TK73 and K4931TK00, characterized as RAPD types K, W, E and X, respectively; lanes 8 to 12, reference strains 6/85, HF51, ts-11, F and R, with RAPD types M, N, L, B and C; lane 13, negative control; lane 14, molecular mass marker. (b) Lane 15, reference strain HF51; lanes 16 to 20, isolates K4158CTK96, K4110ATK96, K4110BTK96, K4110FTK96 and K4181BTK96, characterized as RAPD type O; lanes 21 to 23, isolates K5029BCK00, K5037ACK00 and K5039HCK00, characterized as RAPD type Z; lanes 24, 25, 26 and 27, reference strains 6/85, t-s11, R and F, respectively; lane 28, negative control; lane 29, molecular mass marker.

GTS analysis

Dendrograms were constructed from individual and multiple gene sequence alignments. A total of six dendrograms constructed for GTS analysis are available with the online version of this paper. Dendrograms 1-*gapA*, 2-MGA_0319, 3-*mgc2* and 4-*pvpA* were constructed from individual gene alignments, and dendrograms 5-*mgc2*/*pvpA* and *6-gapA*/MGA_0319/*mgc2*/*pvpA* were constructed from alignments of combined *mgc2* and *pvpA* sequences or all four targeted genes, respectively. Table 2 shows the *M. gallisepticum* strains and isolates listed by RAPD type with the corresponding sequence types for individual and multiple GTS analysis.

GTS of multiple genes and RAPD analysis enabled correlation of epidemiologically linked isolates. Twenty-three of the fifty-four (43 %) USA isolates were epidemiologically related to several *M. gallisepticum* poultry outbreaks and were differentiated by both RAPD and GTS analysis. Poultry outbreak related isolates were separated into six RAPD types J, O, P, U, Y and Z, and into ten different sequence types, VIIa, VIIb, XIa, XIb, Xa, XVa, XVb, XIIIa, XIIIb and XIVa, as shown by *gapA*/MGA_0319/*mgc2*/*pvpA* GTS analysis (Table 2).

Table 2. RAPD and GTS analysis of *M. gallisepticum* reference strains and field isolates

Ref. strain or isolate*	Origin	RAPD type	GTS type†					
			Single gene				Multiple genes	
			gapA	MGA_0319	*mgc2*	*pvpA*	*mgc2/pvpA*	*gapA*/MGA_0319/*mgc2/pvpA*
A5969-CK55	USA	A	Ic	Ia	Ia	Ia	If	Ia
F-CK58	CA, USA	B	IIa	IIa	IIa	IIb	IIa	IIa
K4781ATK99	VA, USA	B	IIb	IIa	IIa	IIb	IIa	IIb
K5058ETK01	VA, USA	B	IIb	IIa	IIa	IIb	IIa	IIb
K5104TK01	VA, USA	B	IIa	IIa	IIa	IIb	IIa	IIa
R-CK60	GA, USA	C	IIIc	IIIa	IIIb	IIIa	IIIb	IIIa
S6-TK61	CA,USA	D	IVa	IVa	Ia	IVd	Ie	IVa
K435TK73	GA, USA	E	Ia	IIIa	Ic	IIIa	IIIc	IIIc
K503CK74	USA	G	IIc	VIa	IIb	VIa	VIa	VIa
K703CK75	USA	H	IId	VIb	IIc	VIa	VIb	VIb
K730CK75	USA	I	IIe	VIc	IId	VIa	VIc	VIc
K2101CK84	CO, USA	J	Va	Va	IVa	IIIa	IIIa	VIIa
K4385TK97	CO, USA	J	Va	Va	IVa	IIIa	IIIa	VIIa
K4414ATK97	CO, USA	J	Vb	Va	IVa	IIIa	IIIa	VIIb
K4902TK00	CO, USA	J	Va	Va	IVa	IIIa	IIIa	VIIa
K3020TK90	CA, USA	K	IIIc	IIIa	IIIb	Va	VIIa	IIIb
ts-11‡	Australia	L	Va	Ia	VIa	IIa	VIIIa	VIIIa
K4688CCK98	NC, USA	L	Va	Ia	VIa	IIa	VIIIa	VIIIa
K4688FCK98	NC, USA	L	Va	Ia	VIa	IIa	VIIIa	VIIIa
K4688GCK98	NC, USA	L	Va	Ia	VIa	IIa	VIIIa	VIIIa
K5109BCK01	PA, USA	L	Va	Ia	VIa	IIa	VIIIa	VIIIa
K5109DCK01	PA, USA	L	Va	Ia	VIa	IIa	VIIIa	VIIIa
K5155CCK01	PA, USA	L	Va	Ia	VIa	IIa	VIIIb	VIIIb
AU083CK80	Australia	L	Va	Ia	VIa	IIf	VIIIa	VIIIa
6/85‡	USA	M	Va	VIIa	VIIa	VIIa	IXa	IXa
K3944TK95	NC, USA	M	Va	VIIa	VIIa	VIIa	IXa	IXa
K4029TK95	NE, USA	M	Va	VIIa	VIIa	VIIa	IXa	IXa
K4043TK95	NE, USA	M	Va	VIIa	VIIa	VIIa	IXa	IXa
K4421ATK97	MI, USA	M	Va	VIIa	VIIa	VIIa	IXa	IXa
K4423BTK97	MI, USA	M	Va	VIIa	VIIa	VIIa	IXa	IXa
K4465TK97	OH, USA	M	Va	VIIa	VIIa	VIIa	IXa	IXa
K5111ATK01	VA, USA	M	Va	VIIa	VIIa	VIIa	IXa	IXa
K3839HF94	MD, USA	N	Va	IVa	IIIa	IVb	Va	Va
HF51-95	GA, USA	N	Va	IVa	IIIa	IVb	Va	Va
K4013HF95	PA, USA	N	Va	IVa	IIIa	IVb	Va	Va
K4094HF96	TN, USA	N	Va	IVa	IIIa	IVb	Va	Va
K4366GF97	SC, USA	N	Va	IVa	IIIa	IVb	Va	Va
K4409HF97	TX, USA	N	Va	IVa	IIIa	IIIb	Va	Va
K4593HF98	MD, USA	N	Va	IVa	IIIa	IVb	Va	Va
K5054TK01	IN, USA	N	Va	IVa	IIIa	IVb	Va	Va
K4110ATK96	NC, USA	O	IIIb	IVa	Va	IVa	Xa	XIb
K4110BTK96	NC, USA	O	IIIa	IVa	Va	IVa	Xa	XIa
K4110FTK96	NC, USA	O	IIIb	IVa	Va	IVa	Xa	XIb
K4158CTK96	MO, USA	O	IIIa	IVa	Va	IVa	Xa	XIa
K4181BCK96	AR, USA	O	IIIa	IVa	Va	IVa	Xa	XIa
K4181CCK96	AR, USA	P	Va	Ia	IVa	IVa	Vc	XIIIb
K4246TK96	AR, USA	P	Va	Ia	Ib	IVa	Ia	XIIIa
K4280CK96	MO, USA	P	Va	Ia	Ib	IVa	Ia	XIIIa
K4311TK96	AR, USA	P	Va	Ia	Ib	IVa	Ia	XIIIa
K4236TK96	VA, USA	Q	Va	Ib	VIIa	IVa	IXb	IXb

Table 2. cont.

Ref. strain or isolate*	Origin	RAPD type	GTS type†					
			Single gene				Multiple genes	
			gapA	MGA_0319	*mgc2*	*pvpA*	*mgc2/pvpA*	*gapA*/MGA_0319/*mgc2/pvpA*
K4355CK96	CA, USA	T	IVb	IVa	VIb	IVd	XIc	IVb
K4649ATK98	CO, USA	U	Ia	IVa	IVa	VIIIa	Vb	Xa
K4649BTK98	CO, USA	U	Ia	IVa	IVa	VIIIa	Vb	Xa
K4669ATK98	CO, USA	U	Ia	IVa	IVa	VIIIa	Vb	Xa
K4657CK98	GA, USA	V	IIIa	VIIb	Vb	IIa	IVb	IIIe
K4705CK99	AR, USA	W	Id	Ib	Ib	IVf	Id	Ib
K4931TK00	VA, USA	X	Va	IIIb	VIa	IVa	XIb	XIIa
K5011TK00	MD, USA	Y	IIIa	VIIIa	Ia	IVa	Ib	XVa
K5027BTK00	IN, USA	Y	IIIa	VIIIa	Ia	IVe	Ic	XVb
K5033ATK00	IN, USA	Y	IIIa	VIIIa	Ia	IVa	Ib	XVa
K5033FTK00	IN, USA	Y	IIIa	VIIIa	Ia	IVa	Ib	XVa
K5029BCK00	PA, USA	Z	Va	Va	VIa	IVc	XIa	XIVa
K5037ACK00	PA, USA	Z	Va	Va	VIa	IVc	XIa	XIVa
K5039HCK00	PA, USA	Z	Va	Va	VIa	IVc	XIa	XIVa
K5120TK01	GA, USA	AA	Va	Ia	VIa	IVa	XIb	XIIb
AU043CK94	Australia	AB	Va	IVa	VIa	VIIb	XId	IVc
AU022CK96	Australia	AC	Ib	IVa	Id	IIc	IVa	IIId
AU019CK97	Australia	AD	Va	Xa	VIc	IId	VIIIc	VIIIc
AU169CK99	Australia	AE	Va	IVa	VIa	IIe	VIIId	VIIId
KSC-3CK99	Israel	AF	VIa	IXb	VIIIb	IXb	XIId	XVId
UHP-1CK99	Israel	AG	Ib	IXb	VIIIc	IXa	XIIf	XVIf
BRT-14CK00	Israel	AH	VIa	IXa	VIIIa	IXa	XIIa	XVIa
YBS-2TK00	Israel	AI	VIa	IXb	VIIIa	IXb	XIIe	XVIe
DSD-6TK00	Israel	AJ	VIa	IXa	VIIIa	IXa	XIIa	XVIa
KS-2CK01	Israel	AK	VIa	IXa	VIIIa	IXb	XIIb	XVIb
MK-8CK01	Israel	AL	VIa	IXa	VIIIa	IXb	XIIc	XVIc
OR-2CK01	Israel	AM	VIa	IXa	VIIIa	IXa	XIIa	XVIa
No. of types§		36	17	16	20	22	38	40

*CK, chicken; TK, turkey; HF, house finch; GF, goldfinch.

†Sequence types are designated with a roman number (I to XV) representing different gene sequence clusters followed by lower-case letters (a to f) representing different gene sequence types within a cluster with identities $<100\,\%$.

‡Live-attenuated vaccine strains introduced in the field during the early 1990s.

§Total number of RAPD and sequence types.

Sixteen of the fifty-four (30 %) USA field isolates were characterized as similar to vaccine strains F, ts-11 or 6/85 by RAPD (types B, L and M) and GTS analysis of multiple genes (Table 2). Epidemiological evidence indicated that some of the isolates within the RAPD group M (K3944TK95, K5111ATK01) and RAPD group L (K5109BCK01, K5109DCK01, K5155CCK01) originated from flocks vaccinated with 6/85 and ts-11, respectively. However, from the sixteen vaccine-like isolates, three F-strain-like (K4781ATK99, K5058ETK01, K5104TK01), three ts-11-like (K4688CKC, K4688CKF, K4688CKG) and five 6/85-like (K4029TK95, K4043TK95, K4421ATK97, K4423BTK97, K4465TK97) isolates originated from non-vaccinated flocks. GTS analysis revealed that F-strain-like isolates K4781ATK99 and K5058ETK01 exhibited the same nucleotide polymorphism in the *gapA* gene sequence as F strain p124 (Table 2, sequence types IIa and IIb).

All six house finch isolates (RAPD type N), with the exception of isolate K4409HF97, were identical by RAPD and GTS analysis of individual and multiple genes (Table 2). The only difference between isolate K4409HF97 and the other five finch isolates was a 231 nt deletion in the *pvpA* gene (sequence type IIIb). Nonetheless this isolate was identified as sequence type Va, similar to the other finch isolates, by GTS analysis of multiple genes (Table 2).

Even though the Israeli isolates did not share 100 % sequence identity with each other, cluster analysis consistently resulted in the segregation of these isolates into a separate cluster away from the USA and Australian isolates. Israeli isolates were differentiated as two sequence types by *gapA* GTS (VIa and Ib), two sequence types by MGA_0319 GTS (IXa and IXb), two sequence types by *pvpA* GTS (IXa and IXb), three sequence types by *mgc2* GTS (VIIIa, VIIIb, VIIIc), five sequence types by *mgc2*/*pvpA* GTS, six sequence types by *gapA*/MGA_0319/*mgc2*/*pvpA* GTS analysis, and eight RAPD types (AF–AM) (Table 2).

The Australian isolates could be differentiated from each other and from the remaining isolates by both RAPD and GTS analysis. Isolate AU083CK80, the parent strain from which the ts-11 vaccine was derived by mutagenesis (Soeripto *et al.*, 1989), was identical to vaccine ts-11 by GTS and RAPD analysis. The remaining four Australian isolates (AU043CK94, AUO22CK96, AU019CK97, AU169CK99) were more similar to isolates from the USA than to the isolates from Israel. The four Australian isolates were readily differentiated by *pvpA* GTS analysis (types VIIb, IIc, IId, IIe), by *mgc2*/*pvpA* (types XId, IVa, VIIIc, VIIId), by *gapA*/MGA_0319/*mgc2*/*pvpA* (types IVc, IIId, VIIIc, VIIId) and by RAPD analysis (RAPD types AB–AE) (Table 2).

Table 3. Gene size polymorphism and RAPD analysis of *M. gallisepticum* isolates

PCR product size (bp)		No. of isolates*	RAPD type†
pvpA	*mgc2*		
665	857	4	J
	854	13	A, E, L, V, AD, AE
	824	1	C
	761	1	AC
606	857	11	N, P‡, U
	854	21	D, O, P, T, W, X, Y, Z, AA, AB
	839	1	K
	761	9	M, Q
578	857	3	G, H, I
	854	8	AF, AG, AH, AI, AJ, AK, AL, AM
437	857	5	B, N§

*Total number of isolates with specific *pvpA*/*mgc2* PCR product size.
†RAPD types corresponding to the different *pvpA*/*mgc2* PCR product size combinations.
‡Isolate K4181CCK96.
§Isolate K4409HF97.

Gene size polymorphism analysis

Gene size polymorphism was not detected among the *gapA* and CDS MGA_0319 amplification products of the 77 *M. gallisepticum* isolates: all isolates yielded 332 and 590 bp PCR products with the gapA and lp primers, respectively. However, as previously reported (Boguslavsky *et al.*, 2000; Liu *et al.*, 2001; Pillai *et al.*, 2003), the *pvpA* gene exhibited size polymorphism, with PCR products of 437, 578, 606 and 665 bp detected among *M. gallisepticum* reference strains and isolates examined (Table 3). 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). Previous sequence analysis of *M. gallisepticum* reference strains R (RAPD type C) and A5969 (RAPD type A) demonstrated that these two strains possess both complete DR sequences, and yield a 665 bp PCR product. Isolates from the USA, characterized as RAPD types E, J, L and V, and Australian isolates, characterized as RAPD types AC, AD and AE, all produced a 665 bp PCR product where the complete *pvpA* gene DR region is present, as confirmed by sequencing analysis. Forty-two of the fifty-four USA isolates, characterized as RAPD types D, K, M–Q, T, U, W–Z and AA, and Australian isolate AU043CK94 (RAPD type AB) yield a 606 bp PCR product with the *pvpA* primers (Table 2). Sequence analysis indicated that all these isolates have a deletion of 59 nt located between the two DR sequences. Gene size polymorphism of the *pvpA* gene has been previously reported for *M. gallisepticum* atypical strains K503CK74 (RAPD type G), K703CK75 (RAPD type H) and K730CK75 (RAPD type I) (Boguslavsky *et al.*, 2000). In this study we found that all Israeli isolates (RAPD types AF–AM) share the *pvpA* size polymorphism with the USA atypical *M. gallisepticum* strains. However, the full *pvpA* gene sequence of one Israeli isolate as compared to the USA atypical *M. gallisepticum* strain K703CK75 indicated that the latter has an additional 3 nt insertion near the DR region (Boguslavsky *et al.*, 2000) not included by this GTS analysis. RAPD type B isolates, including vaccine strain F and house finch isolate K4409HF97 (RAPD type N), are among the other *M. gallisepticum* strains for which *pvpA* size polymorphism has been previously reported (Boguslavsky *et al.*, 2000; Liu *et al.*, 2001).

Gene size polymorphism was also evident among the *mgc2* PCR products (Table 3). The most obvious size polymorphism was observed for RAPD type M isolates, including vaccine strain 6/85, isolate K4236TK96 (RAPD type Q) and isolate AU022CK96 (RAPD type AC); all these isolates produced a 761 bp PCR product with a 63 nt deletion in the DR region of the MGC2 protein carboxy-terminus (Hnatow *et al.*, 1998). Forty-two isolates yielded a PCR product of 854 bp, twenty-three isolates yielded a PCR product of 857 bp, reference strain R yielded a 824 bp PCR product, while isolate K3020TK90 (RAPD type K) yielded a PCR product of 839 bp (Table 3).

In order to determine if size polymorphism of both the *pvpA* and the *mgc2* genes was feasible for typing *M. gallisepticum* isolates the *pvpA*/*mgc2* PCR product sizes were compared to the corresponding RAPD types (Table 3). A total of 11 *pvpA*/*mgc2* PCR size combination types were identified. Seven isolates showed unique PCR size combinations that correlated with specific RAPD types. These were

RAPD type J isolates (K2101CK84, K4385TK97, K4424ATK97, K4902TK00), R-strain, RAPD type K isolate (K3020TK90) and RAPD type AC isolate (AU022CK96). On the other hand, a total of 70 isolates belonging to 32 different RAPD types were distributed within the nine remaining *pvpA*/*mgc2* PCR size types (Table 3).

Discrimination index of gene size polymorphism, GTS and RAPD

To evaluate the capability of gene size polymorphism, GTS and RAPD analysis to discriminate between *M. gallisepticum* isolates, the discrimination index was estimated for each genotyping method. GTS analysis of individual genes *gapA*, MGA_0319, *mgc2* and *pvpA*, identified 17, 16, 20 and 22 sequence types, while gene size polymorphism of the *pvpA*/*mgc2* genes identified 11 types. *D* index values of 0·713 (GTS *gapA*), 0·874 (GTS MGA_0319), 0·915 (GTS *mgc2*), 0·920 (GTS *pvpA*) and 0·854 (*pvpA*/*mgc2* PCR size) were estimated for GTS analysis of individual genes and for gene size polymorphism (Table 4). The estimated discrimination indices indicated that individual GTS analysis of *mgc2* and *pvpA* genes provided adequate discriminatory power. However, GTS analysis of multiple genes provided better discriminatory power. GTS analysis of *mgc2*/*pvpA* identified 38 sequence types and GTS analysis of *gapA*/MGA_0319/*mgc2*/*pvpA* identified 40 different sequence types from a total of 77 sequences. Discrimination indices of 0·962 (GTS *mgc2*/*pvpA*) and 0·965 (GTS *gapA*/MGA_0319/*mgc2*/*pvpA*) were estimated for GTS of multiple genes. Genotyping by RAPD analysis identified 36 different pattern types with a *D* index of 0·958 (Table 4).

Overall, the discriminatory power of the GTS method using multiple genes was slightly higher than the discriminatory power of the RAPD method. However, not all *M. gallisepticum* isolates required GTS analysis of multiple genes in order to be genotyped. Thirty-six, thirty-two, twenty-one and fourteen of the seventy-seven isolates were readily differentiated by GTS analysis of individual *pvpA*, *mgc2*, MGA_0319 and *gapA* genes.

DISCUSSION

Reliable methods for the differentiation of *M. gallisepticum* strains play a pivotal role in understanding the epidemiology and spread of the disease because they generate the information necessary to identify and track new outbreaks. Ideally, methods of strain differentiation must have high enough discriminatory power to clearly differentiate unrelated strains, as well as to demonstrate the relationship of isolates from individuals infected through the same source. Genotyping methods should also have a high degree of reproducibility, which is particularly important during construction of reliable databases. Furthermore, genotyping methods should be easy to interpret and rapid to perform. Progress in the molecular biology of *M. gallisepticum* (Razin *et al.*, 1998) and the availability of the complete genome sequence (Papazisi *et al.*, 2003) have driven the idea to evaluate GTS as a typing tool for differentiating *M. gallisepticum* strains.

This study focused on the evaluation of GTS analysis of three known surface-protein genes (*gapA*, *mgc2* and *pvpA*), and one predicted surface lipoprotein (MGA_0319) as a method for genotyping *M. gallisepticum* strains. Sequencing of targeted genes from selected *in vitro* passages of *M. gallisepticum* strains (R, F, S6, ts-11, 6/85) indicated that the gene regions analysed in this study were stable within passages of the same strains. Overall the *gapA* and MGA_0319 gene sequences were more conserved than the *mgc2* and *pvpA* gene sequences. The genetic variability of the *pvpA* gene has been previously documented (Boguslavsky *et al.*, 2000; Liu *et al.*, 2001; Pillai *et al.*, 2003); however, the genetic variability of the *mgc2* gene has not been previously addressed.

Table 4. Comparison of RAPD, GTS and gene size polymorphism genotyping methods of *M. gallisepticum*

Genotyping method	No. of genotypes (*n*)*	Frequent genotype†	No. of isolates‡	Discrimination (*D*) index§
RAPD	36	L, M, N	8	0·958
GTS gapA	17	Va	40	0·713
GTS MGA_0319	16	IVa	27	0·874
GTS mgc2	20	VIa	15	0·915
GTS pvpA	22	IVa	15	0·920
GTS mgc2/*pvpA*	38	Va, IXa	8	0·962
GTS *gapA*/MGA_0319/*mgc2*/*pvpA*	40	Va, IXa	8	0·965
Gene size polymorphism *pvpA*/*mgc2*	11	606/854	21	0·854

*n, total number of genotypes observed for each method.

†Most frequently observed RAPD type, sequence type and PCR size.

‡Number of isolates within the most frequently observed genotype.

§$D = 1 - \Sigma n(n-1)/N(N-1)$, n = number of genotypes, $N = 77$ (total number of isolates analysed).

In addition to nucleotide sequence variability, gene size polymorphism of the *pvpA* and *mgc2* genes was observed among *M. gallisepticum* strains. Notably, variation in the sequences and size of the *pvpA* and *mgc2* genes was mostly located in the proline-rich, surface-exposed carboxy-terminal-encoding region of both genes. Size polymorphism of both *pvpA* and *mgc2* genes was evaluated as a possible strategy to differentiate among *M. gallisepticum* isolates but only seven isolates showed unique PCR size combinations. Furthermore, short nucleotide insertions/deletions were difficult to detect on ethidium bromide stained agarose gels and sequencing of the PCR products was ultimately required. Therefore differentiation of isolates based on PCR size polymorphism of the *pvpA* and *mgc2* genes was not considered as a reliable method to differentiate among *M. gallisepticum* isolates.

Sequence analysis of the four target genes allowed the establishment of sequence clusters that included USA isolates from known poultry outbreaks, isolates closely related to vaccine strains, and isolates from finches. Australian isolates separated into different gene clusters together with USA isolates, while Israeli isolates separated into a complete distinct cluster separate from the USA and Australian isolates.

Some USA isolates were identified as vaccine strains F, ts-11 and 6/85 by GTS analysis of multiple genes. In particular 6/85-like isolates from turkeys have been extensively genotyped by GTS analysis of the same genes presented in this study, and by RAPD analysis using four different primer sets (Kleven *et al.*, 2004). Whether these isolates are 6/85-derived vaccine subpopulations or isolates closely related to the vaccine that evolved independently in the field is still not clear. To precisely determine the relation of these vaccine-like isolates to the commercially available vaccines, complete vaccine genome sequences and further analysis of the genetic stability of live *M. gallisepticum* vaccines in the field are needed.

Six house finch isolates were characterized as identical by RAPD and GTS analysis of individual and multiple genes indicating genetic homogeneity among these isolates, as previously reported (Ley *et al.*, 1997a). This result is in contrast to findings in a recently reported study where 55 house finch isolates, with similar RAPD type, were grouped into 16 different genotypes by sequence analysis of the *pvpA* gene (Pillai *et al.*, 2003). This result indicated that genetic variability among house finch isolates does exist, and this was recognized by examining a larger population of isolates. The identification of turkey isolate K5054TK01 as identical to the house finch isolates verified our earlier reports of a naturally occurring house-finch-like isolate in a turkey breeder flock (Ferguson *et al.*, 2003). This isolate has been characterized by its low pathogenicity in turkeys and chickens, and its potential as a live *M. gallisepticum* vaccine for turkeys and chickens has been evaluated (Ferguson *et al.*, 2004).

Overall, GTS analysis of multiple surface-protein genes was demonstrated to have better discriminatory power than RAPD analysis. The discriminatory power hierarchy, from highest to lowest, for GTS analysis of individual genes was ranked as: *pvpA*, *mgc2*, MGA_0319 and *gapA*. In addition to its proven discriminatory power, the inter-laboratory reproducibility of GTS analysis was validated by comparing sequence results from the *M. gallisepticum* reference strains and the Israeli isolates obtained independently in the laboratories in Israel and the USA.

The identification of gene polymorphism in bacteria by nucleotide sequence analysis of genes encoding antigenic surface proteins jointly with genes encoding housekeeping proteins has been proven useful in the surveillance of pathogenic bacteria (Byun *et al.*, 1999; Maiden *et al.*, 1998; van Loo *et al.*, 2002; Kotetishvili *et al.*, 2003). Particularly multilocus sequence typing (MLST) schemes where data can be stored, analysed and queried through a central web server have permitted local and global epidemiology studies of several infectious agents (Chan *et al.*, 2001).

In this study we have demonstrated that GTS analysis of *M. gallisepticum* surface-protein genes is a reproducible typing method with satisfactory discriminatory power to separate isolates from unrelated outbreaks and to identify closely related isolates. Further development of a *M. gallisepticum* GTS database, including sequences from surface proteins as well as housekeeping genes, will provide a global alternative for typing *M. gallisepticum* isolates, and a resource to understand the evolutionary relationships of this poultry pathogen.

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