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Molecular Variability of the Adhesin-Encoding Gene *pvpA* among *Mycoplasma gallisepticum* Strains and Its Application in Diagnosis

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***Mycoplasma gallisepticum* is an important pathogen of chickens and turkeys that causes considerable economic losses to the poultry industry worldwide. The reemergence of *M. gallisepticum* outbreaks among poultry, the increased use of live *M. gallisepticum* vaccines, and the detection of *M. gallisepticum* in game and free-flying song birds has strengthened the need for molecular diagnostic and strain differentiation tests. Molecular techniques, including restriction fragment length polymorphism of genomic DNA (RFLP) and PCR-based random amplification of polymorphic DNA (RAPD), have already been utilized as powerful tools to detect intraspecies variation. However, certain intrinsic drawbacks constrain the application of these methods. The main goal of this study was to determine the feasibility of using an *M. gallisepticum*-specific gene encoding a phase-variable putative adhesin protein (PvpA) as the target for molecular typing. This was accomplished using a *pvpA* PCR-RFLP assay. Size variations among PCR products and nucleotide divergence of the C-terminus-encoding region of the *pvpA* gene were the basis for strain differentiation. This method can be used for rapid differentiation of vaccine strains from field isolates by amplification directly from clinical samples without the need for isolation by culture. Moreover, molecular epidemiology of *M. gallisepticum* outbreaks can be performed using RFLP and/or sequence analysis of the *pvpA* gene.**

Mycoplasma gallisepticum is one of the major pathogens of domestic poultry, causing significant economic losses to the commercial poultry industry. *M. gallisepticum* infection has a wide variety of clinical manifestations, of which chronic respiratory disease of chickens is the most significant. The pathology associated with this disease is characterized by severe air sac infection where *M. gallisepticum* is the primary pathogen followed by secondary infections with *Escherichia coli* and/or viruses (17). Control of *M. gallisepticum* has been based on eradication of the organism from primary breeder flocks and on maintenance of the mycoplasma-free status in the breeders and breeder progeny by biosecurity of the premises (15). Serological monitoring of a representative sample of the flock is performed periodically, and isolation or DNA-based detection methods (15, 17) confirm suspected infection by *M. gallisepticum*. In recent years, a reemergence of mycoplasma infection has been observed in poultry, possibly due to the practice of placing large poultry populations in small geographical areas under poor biosecurity. This has necessitated a reevaluation of control strategies for *M. gallisepticum* (12).

One of the options as an alternative control method is the use of live *M. gallisepticum* vaccines (12, 28). Three live *M. gallisepticum* vaccines are currently used in many countries worldwide: F (Schering Plough, Kenilworth, N.J.), ts-11 (Bioproperties, Inc. Australia, marketed in the United States by Merial Select Laboratories, Gainesville, Ga.), and 6/85 (In-

tervet America, Millsboro, Del.). The more widespread utilization of *M. gallisepticum* vaccines requires the development of improved detection and differentiation methods in order to assess the efficacy of vaccines in displacing wild-type isolates (18, 26). Currently, the most widely used method to differentiate *M. gallisepticum* strains is a PCR-based randomly amplified polymorphic DNA (RAPD), or arbitrarily primed PCR, analysis (3, 5, 7). This method has been used for identifying vaccine strains in *M. gallisepticum*-vaccinated flocks (18) and for epidemiological studies (19). Due to the random nature of the primer and the low-stringency conditions of the RAPD reaction, this assay requires the use of pure cultures of the target mycoplasma. Isolation of mycoplasmas is expensive, time-consuming, and technically complicated in cases in which nonpathogenic mycoplasma species may overgrow the virulent mycoplasmas. In addition, the isolation process itself may favor the growth of one strain where more than one *M. gallisepticum* subtype may be present. Furthermore, technical factors such as the target DNA/primer ratio may significantly impact the reproducibility of RAPD patterns (27). Progress in the molecular biology of mycoplasmas has been achieved in the last decade, and several surface proteins in virulent mycoplasmas have been described (1, 22). Mycoplasmas exhibit a high degree of phenotypic variation, which is considered a major factor in pathogenicity and chronic infection of the host (22, 23, 24, 29). Changes in the surface topology of *M. gallisepticum* during host infection and the molecular characteristics of several *M. gallisepticum* surface proteins have been described (1, 6, 14, 21), including the recently described putative cytadhesin protein PvpA (2). PvpA is a phase-variable protein recognized by the chicken immune system (14, 30). Structurally, PvpA is a non-

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lipid integral membrane protein with a surface-exposed C-terminal portion (30). The surface-exposed C terminus of PvpA protein has a high proline content (28%) and contains identical direct repeat sequences consisting of 52 amino acids each, designated DR-1 and DR-2 (2). Size variation of the *pvpA* gene was observed among *M. gallisepticum* strains as a result of deletions occurring in the segment encoding the the proline-rich C-terminal region of the protein. Deletions were located exactly within the two direct repeat sequences (2). The presence of proline-rich regions in the surface-exposed C-terminal domains of other pathogenic mycoplasma adhesins (4, 8, 9) suggests an important role of these domains in the function of PvpA as an adhesin (10). Moreover, molecular typing of *M. gallisepticum* strains utilizing the C-terminus-encoding region of the *pvpA* gene may be a relevant target for epidemiological tracking of *M. gallisepticum* isolates.

The main goal of our study was to determine the feasibility of using the variable *pvpA* gene as the target to differentiate *M. gallisepticum* strains through a PCR restriction fragment length polymorphism (PCR-RFLP) assay. To design consensus primers to amplify all *M. gallisepticum* strains, the nucleotide sequence of the C-terminus-encoding region of the *pvpA* gene was determined from 10 reference *M. gallisepticum* strains and 24 field isolates. Various deletions were observed within DR-1 and DR-2, as previously described by Boguslavsky et al. (2). Further differentiation among *M. gallisepticum* reference strains and field isolates was obtained by RFLP using three restriction enzymes. To enhance the sensitivity of the assay, a seminested set of primers was also designed. This assay was used to detect *M. gallisepticum* directly from clinical samples. Amplicons obtained from clinical samples were analyzed according to the RFLP patterns typed with restriction enzymes and further sequenced. This assay accelerates the diagnosis and characterization of *M. gallisepticum* isolates and can be used as a molecular typing tool in order to better understand the epidemiology of *M. gallisepticum* outbreaks.

MATERIALS AND METHODS

Mycoplasma reference strains and isolates. Most mycoplasma strains were obtained from our depository at the Poultry Diagnostic and Research Center (PDRC) in Athens, Ga. The type strains of the avian species *Mycoplasma synoviae*, *Mycoplasma meleagridis*, *Mycoplasma iowae*, *Mycoplasma gallinarum*, *Mycoplasma gallinaceum*, *Mycoplasma iners*, *Mycoplasma imitans*, and *Mycoplasma cloacale* were used for testing the specificity of the PCR test. The origins and biological properties of *M. gallisepticum* strains R, S6, A5969, F, K503, K703, K730, and HF-51 are described elsewhere (31). Vaccine strain ts-11 was obtained from Merial Select (Gainesville, Ga.), and 6/85 was obtained from Intervet America (Millsboro, Del.). Twenty-two field isolates obtained during recent outbreaks (1995 to 1999) and two field isolates from 1973 and 1984 were collected from our depository. Among these, seven isolates were from chickens, eleven were from turkeys, and six were from house finches (*Carpodacus mexicanus*). Table 1 presents a list of the isolates used.

Culture procedures and sample preparations. All avian mycoplasma strains were propagated in Frey's medium (17) with 12% swine serum (FMS) as previously described (5, 6). DNA isolation was performed as follows. One milliliter of mycoplasma broth culture was centrifuged at 13,000 rpm (Eppendorf 5417R; Brinkmann Instruments, Westbury, N.Y.) for 10 min. The cell pellet was then washed twice with 1 ml of 150 mM phosphate-buffered saline (PBS, pH 7.2) and resuspended in a final volume of 20 μl of PBS. The cell suspension was heated in a dry block at 110°C for 10 min and placed on ice for at least 10 min. After boiling, the lysate was centrifuged at 13,000 rpm for 2 min to remove debris. The supernatant containing DNA was collected and stored at −20°C until used.

DNA extraction from clinical samples was prepared by suspending tracheal swabs in 1 ml of PBS (three tracheal swabs per sample) and spinning at 13,000

TABLE 1. Field isolates

Case	Isolate[a]
K435	TK/GA/73/1
K2101	CK/CO/84/1
K3839	HF/MA/94/1
K3944	TK/NC/95/1
K4013	HF/PA/95/1
K4029	TK/NB/95/2
K4043	TK/NB/95/1
K4094	HF/TN/96/1
K4236	TK/VA/96/1
K4355	CK/CA/96/1
K4366	GF/SC/97/1
K4409	HF/TX/97/1
K4421A	TK/MI/97/2
K4423B	TK/MI/97/1
K4465	TK/OH/97/1
K4593	HF/MA/98/1
K4649A	TK/CO/98/1
K4649B	TK/CO/98/2
K4657	CK/GA/98/1
K4669A	TK/CO/98/3
K4688C	CK/NC/99/1
K4688F	CK/NC/99/2
K4688G	CK/NC/99/3
K4705	CK/AK/99/1

[a] Isolate names are in the form avian species/state/year/isolate number. TK, turkey; CK, chicken; HF, house finch; GF, goldfinch.

rpm for 30 min. The supernatant was carefully removed and the resultant cell pellet suspended in 25 μl of PCR-grade H₂O and treated as described above.

Clinical samples. Clinical samples were obtained from the United States and Israel and processed at the PDRC or at the Kimron Veterinary Institute (Bet Dagan, Israel), respectively. Twenty tracheas were obtained upon necropsy from two commercial egg layer flocks which were serologically positive for *M. gallisepticum* infection. Tracheas were opened longitudinally, and mucous material was collected with a swab. Trachea material was cultured on Frey's media following standard isolation procedures. Material was resuspended in 500 μl of PBS for DNA extraction and *pvpA* PCR-RFLP analysis.

Tracheal swab samples from four Israeli broiler breeder flocks suspected of having *M. gallisepticum* infection were collected from live birds. Swabs were cultured on Frey's medium by standard isolation procedure, and DNA extracts for PCR testing were prepared from tracheal swabs by the boiling method as described above.

Primer selection. Initial primers were designed from the *pvpA* gene sequence of R strain (2). Seminested PCR primers were designed from conserved sequences of representative *M. gallisepticum* strains. These primers flank the direct repeat area within the C-terminus-encoding region of the *pvpA* gene. Primer positions are depicted in Fig. 1.

***pvpA* PCR.** Amplification reactions for pure culture samples were performed in a 50-μl reaction volume as follows. Five microliters of 10× PCR buffer containing 1.5 mM MgCl₂, 2 μl of 1 mM deoxynucleoside triphosphate (dNTPs)
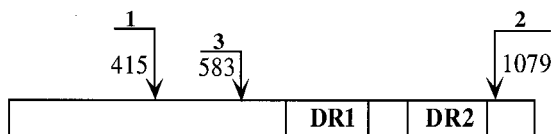

FIG. 1. Schematic diagram of *pvpA* indicating the location of primers for seminested PCR. Primer positions are in relation to the R strain sequence (2). The first reaction used primer 1 (pvpA1F), located at nucleotide positions 415 to 437 (5′GCCAM TCCAACTCAACAAGC TGA3′), and primer 2 (pvpA2R), located at nucleotide positions 1059 to 1081 (5′GGACGTSGTCCTGGCT GGTTAGC3′). The seminested reaction was performed with primer 3 (pvpA3F), located at nucleotide positions 583 to 604 (5′GGTAGTCCTAAGTTATTAGGTC3′), and primer 2 (pvpA2R) as for the first amplification.

(GIBCO BRL, Grand Island, N.Y.), 0.5 μl of outer primers pvpA1F and pvpA2R (50 μM), 0.25 μl of *Taq* DNA polymerase (5 U/μl) (*Taq* PCR; Qiagen Inc., Valencia, Calif.), 40.75 μl of distilled water, and 1 μl of template DNA. All amplifications were performed in a GeneAmp PCR system 2400 (PE Biosystems, Foster City, Calif.). Initially, annealing temperatures for primer extension of 50, 55, and 58°C were tested. The amplification reaction using 55°C as the annealing temperature produced the best amplification yield. This temperature was considered the optimal annealing temperature for *pvpA* primer extension. The thermocycler was programmed as follows: 94°C for 3 min followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and 72°C for 10 min as the final extension step.

In order to increase sensitivity of detection in clinical samples, two amplifications were performed with seminested primers (Fig. 1). The first amplification was carried out in a 25-μl reaction volume consisting of 2.5 μl of 10× PCR buffer containing 1.5 mM MgCl₂, 1 μl of 1 mM dNTPs, 0.25 μl of outer primers (pvpA1F and pvpA2R) (50 μM), 0.13 μl of *Taq* DNA polymerase (5 U/μl), 19.87 μl of distilled water, and 1 μl of template DNA. The first amplification was performed at 94°C for 3 min followed by 20 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and 72°C for 10 min was the final extension step. One microliter from the first amplification was used as the template for the second amplification. The second amplification was performed in 25 μl consisting of 2.5 μl of 10× PCR buffer containing 1.5 mM MgCl₂, 1 μl of 1 mM dNTPs, 0.35 μl of seminested primer (pvpA3F) (50 μM), 0.35 μl of outer primer (pvpA2R) (50 μM), 0.13 μl of *Taq* DNA polymerase (5 U/μl), 19.80 μl of distilled water, and 1 μl of template DNA. The second amplification was performed at the same temperatures and times as the first amplification for 40 cycles.

The detection limit of seminested PCR and single PCR was determined by conducting nine 10-fold serial dilutions from R strain stock culture in FMS. Twenty microliters of each 10-fold dilution was plated on Frey's medium agar plates and incubated at 37°C until colonies were visible. Colonies were counted 5 days after inoculation of the plates. A 1-ml aliquot from each dilution was collected for DNA extraction followed by PCR amplification as described above.

The PCR products were detected by electrophoresis in ethidium bromide-stained agarose gels. Ten microliters of each amplification reaction was loaded on a 1.5% agarose gel containing 0.8 μg of ethidium bromide/ml. Agarose gel electrophoresis was run for 25 min at 110 V, and the gel was visualized under UV light.

Sequence analysis. Amplified products from 10 reference *M. gallisepticum* strains and 24 field isolates were purified with a QIAquick PCR purification kit (Qiagen, Inc.). Purified PCR products were sequenced at the Molecular Genetics Instrumentation Facility, University of Georgia, utilizing automated sequencing with a Prism DyeDeoxy terminator cycle sequencing kit (PE Biosystems). Assembly of sequence contigs and initial multiple-sequence alignments were performed with sequencing project management (SeqMan) and multiple-sequence alignment (MegAlign) programs, respectively (DNASTAR; Lasergene, Inc. Madison, Wis.). Phylogenetic analysis was performed with PAUP 4.0b2 and 4a (Sinauer Associates, Inc., Sunderland, Mass.).

RFLP analysis. Restriction enzyme analysis of sequences was performed with MapDraw (DNASTAR; Lasergene, Inc.). *Pvu*II (Boehringer Mannheim, Indianapolis, Ind.), *Acc*I (New England Biolabs, Inc., Beverly, Mass.), and *Scr*FI (New England Biolabs, Inc.) were identified as suitable for RFLP analysis and utilized for digestion of PCR products. The restriction enzyme digestions were performed in 10-μl reaction mixtures (6.6 μl of H₂O, 1 μl of 10× buffer, 0.4 μl of restriction enzyme, 2 μl of PCR product), and mixtures were incubated at 37°C
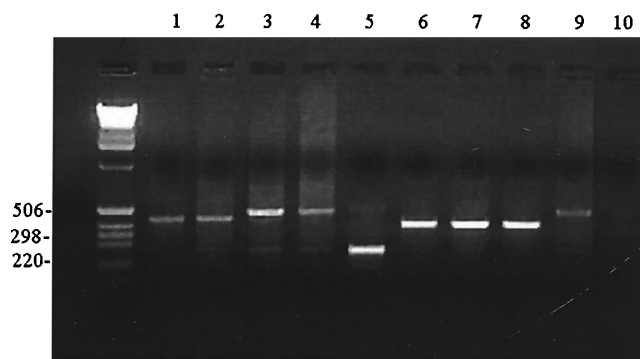

FIG. 2. PCR of *M. gallisepticum* reference with outer primers pvpA 5′ and pvpA 3′. Molecular weight markers (Life Technologies, Rockville, Md.) are in the unmarked lane on the left (sizes on the left are in base pairs). Lane 1, 6/85; lane 2, field isolate (CK/CA/96/1); lane 3, R; lane 4, ts-11; lane 5, F; lane 6, K503; lane 7, K703; lane 8, K730; lane 9, A5969; lane 10, negative control. Size variation of amplification products from 497 bp (lanes 3, 4, and 9) to 267 bp (lane 5) was observed.

for 2 h. The digested PCR products were electrophoresed on a 10% Tris buffer–EDTA gel (Novex, San Diego, Calif.) at a constant 100 V for 50 min. Gels were visualized by silver staining according to the manufacturer's recommendation (Amersham Pharmacia Biotech, Piscataway, N.J.).

16S rRNA gene PCR. An *M. gallisepticum* PCR test was performed with the 16S rRNA primers MG13 and MG14 (13), using 5 μl of DNA extracted as described above. In addition to serological testing and isolation, this PCR test is used routinely in the laboratory at the Kimron Veterinary Institute and at the PDRC to determine the *M. gallisepticum* status of chicken and turkey breeder flocks.

RESULTS

Amplification of the *pvpA* C-terminus-encoding region. Amplification of the *pvpA* C-terminus-encoding region demonstrated size differences of PCR products among *M. gallisepticum* reference strains, as previously reported by Boguslavsky et al. (2). Amplification product size polymorphism was also observed within field isolates analyzed. Size variation of PCR products for reference strains and field isolates was confirmed by nucleotide sequencing analysis. Amplification product sizes for *M. gallisepticum* reference strains ranged from 267 to 497 bp (Fig. 2; Table 2). A PCR product with the expected size of 497 bp was observed for strains R, ts-11, and A5969 (Fig. 2, lanes 3, 4, and 9, respectively); a 437-bp PCR product was ob-

TABLE 2. RFLP patterns of *M. gallisepticum* reference strains

Strain	PCR size[a]	Fragment sizes			RFLP pattern
		*Pvu*II	*Acc*I	*Scr*FI	
R	497	231 + 217 + 49	231 + 156 + 110	240 + 183 + 74	A
ts-11	497	231 + 217 + 49	341 + 156	240 + 183 + 74	B
A5969	497	448 + 49	341 + 156	240 + 183 + 74	C
6/85	437	217 + 120 + 51 + 49	Not cut	363 + 74	D
S6	437	217 + 120 + 51 + 49	Not cut	228 + 135 + 74	E
HF-51	437	217 + 171 + 49	Not cut	228 + 135 + 74	F
K503	410	231 + 130 + 49	Not cut	Not cut	G
K703	410	231 + 130 + 49	Not cut	Not cut	G
K730	410	231 + 130 + 49	Not cut	Not cut	G
F	267	217 + 50	Not cut	193 + 74	H

[a] Size in base pairs, estimated from molecular weight marker and sequence analysis.

TABLE 3. RFLP and sequencing analysis of the C-terminus-encoding region of the *pvpA* gene from field isolates

Field strain	PCR product size[a]	RFLP group[b]	% Sequence identity[c]	
			Nucleotide	Amino acid
TK/GA/73/1	497	A	99.8	99.4
CK/CO/84/1	497	A	100	100
CK/GA/98/1	488	B	98.5	96.9
CK/NC/99/1	497	B	100	100
CK/NC/99/2	497	B	100	100
CK/NC/99/3	497	B	100	100
TK/MI/97/1	437	D	100	100
TK/MI/97/2	437	D	100	100
TK/NB/95/1	437	D	100	100
TK/NB/95/2	437	D	100	100
TK/OH/97/1	437	D	100	100
TK/NC/95/1	437	D	100	100
CK/CA/96/1	437	E	100	100
TK/VA/96/1	437	F	98.8	97.9
TK/CO/98/1	437	F	98.1	96.5
TK/CO/98/2	437	F	98.1	96.5
TK/CO/98/3	437	F	98.1	98.8
HF/MA/94/1	437	F	100	100
HF/PA/95/1	437	F	100	100
HF/TN/96/1	437	F	100	100
GF/SC/97/1	437	F	100	100
HF/MA/98/1	437	F	100	100
HF/TX/97/1	266	H	96.1	91.9
CK/AK/99/1	437	I	98.4	96.5

[a] Size in base pairs, estimated from molecular weight marker and sequence analysis.

[b] RFLP groups correspond to *M. gallisepticum* reference strains as follows: A, R; B, ts-11; D, 6185; E, S6; F, HF-51; H, F.

[c] Sequence identity with corresponding RFLP group *M. gallisepticum* reference strain.

tained for vaccine strain 6/85 and a field isolate (CK/CA/96/1) (Fig. 2, lanes 1 and 2) and for challenge strain S6 and finch isolate HF-51 (data not shown). Atypical strains K503, K703, and K730 produced an amplicon of 410 bp (Fig. 2, lanes 7 and 8). A 267-bp amplicon was obtained for strain F (Fig. 2, lane 5).

Size variation of PCR products was also observed among field isolates (Table 3). Like 6/85 and S6, 18 of the 24 field isolates analyzed produced a 437-bp amplicon; five isolates produced a 497-bp amplicon, and isolates CK/GA/98/1 and HF/TX/97/1 produced amplicons of 488 and 266 bp, respectively. Size variation of PCR products was confirmed by nucleotide sequencing analysis.

RFLP and sequencing analysis of *M. gallisepticum* reference strains. The C-terminus-encoding region of the *pvpA* gene was sequenced for 10 *M. gallisepticum* reference strains. Multiple alignments of *M. gallisepticum* reference strain sequences indicated that variation among PCR products' relative mobilities was attributed to different size deletions at the end of the *pvpA* gene corresponding to the C terminus, as previously described by Boguslavsky et al. (2). The precise location of the deletions allowed the design of three conserved primers (pvpA1F, pvpA2R, and pvpA3F) (Fig. 1) utilized in a seminested PCR amplifying all the strains tested. In addition to size variation of PCR products, nucleotide sequence differences among *M. gallisepticum* reference strains produced different RFLP patterns. Digestion of *M. gallisepticum* reference strains with *Acc*I,

*Pvu*II, and *Scr*FI produced eight distinct pattern combinations. The exact sizes of the fragments generated from digested PCR products are listed in Table 2. *M. gallisepticum* R, ts-11, A5969, HF-51, atypical strains (K503, K703, and K730), and F were readily differentiated into six distinct patterns designated A, B, C, F, G, and H, respectively, with *Pvu*II and *Acc*I. A third restriction enzyme, *Scr*FI, was used to differentiate 6/85 and S6, and the patterns were identified as D and E, respectively (Table 2).

RFLP and sequence analysis of field isolates. The C-terminus-encoding regions of *pvpA* for 24 field isolates were compared by RFLP and sequence analysis. RFLP analysis classified the field isolates into seven of the eight RFLP groups described in Table 2 for *M. gallisepticum* reference strains. Field isolates were grouped as follows (Table 3): two isolates were classified as RFLP group A, represented by challenge strain R; four chicken isolates had RFLP pattern combinations similar to group B, represented by vaccine strain ts-11; six isolates were classified as RFLP group D, represented by vaccine strain 6/85; one isolate was placed within RFLP group E, represented by strain S6; nine isolates, four from turkeys and five from finches, were characterized as RFLP group F, represented by finch strain HF-51; one isolate showed RFLP patterns similar to vaccine strain F, classified as group H. Isolate CK/AK/99/1 produced a unique RFLP pattern (I), a combination not observed for any of the *M. gallisepticum* reference strains or field isolates analyzed.

A perfect correlation was observed between RFLP and sequencing analysis of the amplicon produced by the seminested PCR for 16 field isolates in which a 100% sequence identity was observed between the field isolate and its corresponding *M. gallisepticum* reference strain RFLP group (Tables 2 and 3). One isolate in RFLP group A showed 100% sequence identity to challenge strain R. Three of the four isolates identified as RFLP group B showed 100% identity to vaccine strain ts-11. Six of the seven isolates identified as RFLP group D showed 100% sequence identity to vaccine strain 6/85. One isolate in RFLP group E had 100% sequence identity to S6, in agreement with the results of *Scr*FI RFLP analysis. Five of the nine isolates within RFLP group F, isolated from finches, showed 100% identity to house finch isolate HF-51.

For the remaining eight field isolates, it was observed that the sequence of *pvpA* corresponding to the C-terminal region was not identical to that of the corresponding RFLP group. Sequence analysis of these isolates showed sequence divergences ranging from 99.8 to 96.1% for nucleotides and 99.4 to 91.9% for deduced amino acids (Table 3). Molecular grouping of *M. gallisepticum* isolates by RFLP was further confirmed by phylogenetic analysis of the *pvpA* gene sequences. Figure 3 shows a dendrogram constructed from the C-terminus-encoding end of the *pvpA* gene. Sequences were distributed in seven distinctive groups that correspond to the RFLP groups A, B, C, D, E, F, and G (Fig. 3). A discrepancy between phylogenetic and RFLP analysis was observed for the finch isolate HF/TX/97/1. This isolate, in contrast to the other finch isolates analyzed, produced a PCR product of the same size as vaccine strain F and was grouped by RFLP with this strain (RFLP group H). However, nucleotide sequence comparison indicated that HF/TX/97/1 was most closely related to other finch isolates rather than to F strain (Fig. 3). Further comparison of
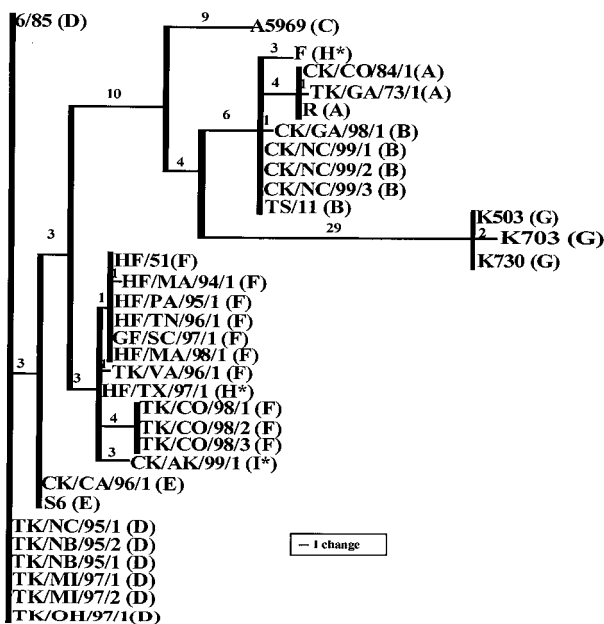
FIG. 3. Phylogentic analysis of the C-terminus-encoding portion of *pvpA* of *M. gallisepticum* reference strains and field isolates. The dendrogram was constructed using parsimony analysis in a full heuristic search with 500 bootstrap resampling replicates. The RFLP group for each respective isolate is indicated in parentheses. Phylogenetic analysis produced seven distinct groups, confirming RFLP grouping: A, B, C, D, E, F, and G. Discrepancies between phylogenetic and RFLP analysis were observed for isolates in groups H* and I*.
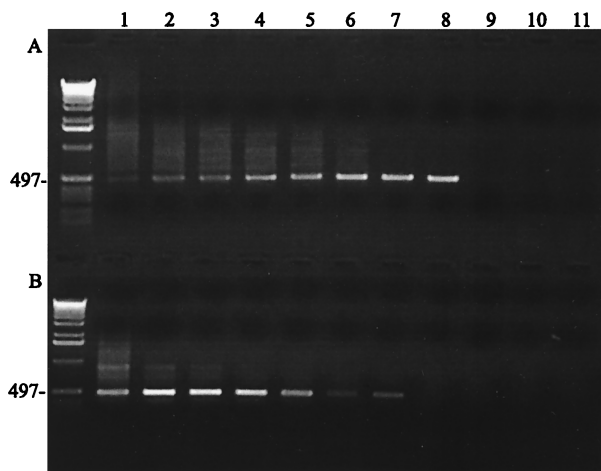

FIG. 4. Sensitivity of seminested PCR versus single PCR. (A) Seminested PCR. Serial dilutions of R strain amplified with seminested primers. Lanes 1 to 10 represent dilutions from 10^{-1} through 10^{-10}. Lane 11 is the negative control. Lane 1, 5×10^8 CFU; lane 2, 5×10^7 CFU; lane 3, 5×10^6 CFU; lane 4, 5×10^5 CFU; lane 5, 5×10^4 CFU; lane 6, 5×10^3 CFU; lane 7, 5×10^2 CFU; lane 8, 5×10^1 CFU. (B) Single PCR with inner primers following the same serial dilutions. Both PCR systems produced a 497-bp band as expected.

the deduced amino acid sequences of finch isolate HF/TX/97/1 and vaccine strain F indicated four amino acid substitutions (data not shown), resulting in amino acid sequence homology of only 91.9%. between the two strains (Table 3). Comparison of the deduced amino acid sequences of finch isolate HF/TX/97/1 and finch strain HF-51 indicated an amino acid sequence homology of 96.3% (data not shown).

Specificity and sensitivity of *pvpA* PCR. Single and seminested amplifications with the three *pvpA* primers were performed against DNA from eight avian mycoplasma species, as indicated in Materials and Methods, including *M. gallinarum*, and *M. gallinaceum*, common normal flora mycoplasmas of the upper respiratory track of chickens. *M. gallisepticum* was the only mycoplasma amplified by *pvpA* primers (data not shown).

Sensitivity of single PCR versus seminested PCR was evaluated by comparing detection limits of both assays. Tenfold serial dilutions were performed from R strain stock culture with an initial titer of 5×10^8 CFU/ml (Fig. 4). A single amplification with primers pvpA3F and pvpA2R detected 500 CFU/ml (Fig. 4B), whereas the detection limit after a second amplification with primers pvpA3F and pvpA2R reached 50 CFU/ml (Fig. 4A), achieving a 10-fold increase in the detection limit.

Clinical samples. Because of the increase in sensitivity achieved by the seminested PCR, this assay was used to analyze clinical samples. Six of 20 samples from two U.S. layer flocks from a common operation tested positive. One positive sample was from flock A, and the remaining five positive samples were from flock B. None of the PCR products were sen-

sitive to *Acc*I digestion, and RFLP group E (Table 3) was observed for the six amplicons when they were digested with *Pvu*II. Sequences obtained from these amplicons showed 100% identity with field isolate TK/VA/96/1 (Table 3). Culture results from tracheal samples were negative for *M. gallisepticum* as well as saprophytic mycoplasma species, which may interfere with the isolation attempt. Since tracheal levels of *M. gallisepticum* in chronically infected birds are often very low (11), the number of viable cells removed from the trachea by swabbing may have been too low to be detected by isolation.

To further compare the sensitivity of *pvpA* PCR in clinical samples, tracheal swabs from three commercial breeder flocks from Israel were tested by three methods: *pvpA* seminested PCR, rRNA-based *M. gallisepticum* PCR (13), and isolation. All four flocks were positive for *M. gallisepticum* by both PCR tests, although the number of individual samples showing a positive reaction differed for the two tests (Table 4). *M. gallisepticum*, *M. synoviae*, and *M. gallinarum* were isolated from

TABLE 4. Comparative analysis of clinical samples from commercial broiler-breeder flocks

Flock age (wk)	No. of positive samples/no. tested by:		Isolation
	M. gallisepticum PCR[a]	*pvpA* PCR[b]	
50	8/10	4/10	*M. gallisepticum, M. synoviae, M. gallinarum*
58	10/10	1/10	NT[c]
58	4/4	4/4	NT
40	5/5	2/5	*M. gallisepticum, M. synoviae, M. gallinarum*

[a] PCR using primers MG13 and MG14, directed to the rRNA gene of *M. gallisepticum*.
[b] Seminested *pvpA* PCR.
[c] NT, not tested.

two flocks tested. Testing of three other chicken and turkey breeder flocks containing *M. synoviae*, *M. gallinarum*, and/or *M. meleagridis* but negative for *M. gallisepticum* were negative in both PCR tests (data not shown).

DISCUSSION

Sequence analysis of the whole *pvpA* gene of reference *M. gallisepticum* strains revealed that different-size deletions are located within DR-1 and DR-2 of the C-terminus-encoding region of *pvpA* (2). In this study we demonstrated by sequencing of the genomic region amplified by the seminested *pvpA* PCR primers that the *pvpA* C-terminal deletions are present also among *M. gallisepticum* strains currently circulating in the field. The largest deletion observed was that of a 230-bp fragment in vaccine strain F. An amplicon of the same size was observed for finch isolate HF/TX/97/1 (Table 3). Differing from isolates with this large deletion were atypical *M. gallisepticum* strains (K503, K703, and K730), with an amplicon of 410 bp. This amplicon had six small deletions scattered in the region of *pvpA* corresponding to the C terminus (2; additional data not shown). More significant yet, 16 of the 24 field isolates analyzed that produced a 437-bp amplicon shared a similar 60-bp deletion with *M. gallisepticum* representative strains 6/85, S6, and HF-51 (Table 2). Based on the deduced amino acid sequence, the deletion extends from amino acid position 88, located outside DR-2, to amino acid position 107, located within DR-2 (data not shown). However, not all field isolates had deletions within the *pvpA* gene. In common with representative strains R, A5969, and vaccine strain ts-11, six field isolates produced the expected 497-bp amplicon where no deletions were observed within the C-terminus-encoding *pvpA* region. In addition to PCR product length polymorphism, nucleotide differences allowed differentiation of representative *M. gallisepticum* strains and field isolates by RFLP of the amplification products. *M. gallisepticum* strains were characterized in seven RFLP groups, whereas field isolates were categorized within five of these seven groups and an additional unique group (Tables 2 and 3).

An important application of this method will be to identify specific *M. gallisepticum* strains and, in particular, to detect the presence of vaccine strains and to monitor their capacity to displace field strains during *M. gallisepticum* vaccination programs. Among analyzed field isolates, six turkey and four chicken isolates were grouped by RFLP as vaccine strains 6/85 and ts-11, respectively (Table 3). These results were confirmed by sequence analysis. A 100% sequence identity was observed among the turkey isolates and vaccine strain 6/85 and between three of the four chicken isolates and vaccine strain ts-11.

The identification of 13 vaccine strains among the 24 field isolates tested in this study was not surprising, since these samples were specifically selected from flocks with previous vaccination history or previous exposure to other vaccinated birds. The presence of vaccine strains in these flocks was suggested on the basis of clinical data and the identification of vaccine strains by the RAPD molecular typing used in our laboratory (S. H. Kleven and V. Leiting, unpublished data). RAPD analyses have been utilized previously to identify and determine the prevalence of vaccine strains ts-11 and 6/85 in experimental (18) and commercial (26) vaccinated flocks.

An outbreak of conjunctivitis in house finches (*Carpodacus mexicanus*) caused by *M. gallisepticum* was first reported during early 1994 (16, 20). Since the beginning of this outbreak, other wild bird species, such as American goldfinches (*Carduelis tristis*) and blue jays (*Cyanocitta cristata*), have been infected (16). RAPD typing of representative isolates from different bird species and geographical regions indicated the presence of a single type (19). In this study, RFLP and sequence analysis of finch isolates from different states demonstrated that sequences among isolates were identical with the exception of HF/TX/97/1. Although isolate HF/TX/97/1 was previously characterized as having an RAPD pattern identical to those of the other finch isolates (S. H. Kleven, unpublished data), this isolate had a 230-bp deletion, and based on RFLP, it was grouped with vaccine strain F. However, sequence analysis demonstrated that this isolate is more closely related to finch isolate HF-51 than to vaccine strain F. The *pvpA* gene divergence among HF/TX/97/1 and other finch isolates suggests that more than one strain of *M. gallisepticum* has been circulating among the finch population during the outbreak. Although free-flying birds have not been strongly implicated in the transmission of pathogenic mycoplasmas to commercial poultry, the relationship between poultry and finch isolates needs to be further analyzed (25).

Characterization of other chicken and turkey isolates was also more reliably obtained by sequencing than by size and RFLP grouping alone. For instance, the isolates TK/VA/96/1 and TK/CO/98/1, -2, and -3 (Table 3) have a *pvpA* C-terminus-encoding region closely related to that of finch isolates, suggesting that finch and poultry isolates may have a common ancestor. However, complete sequencing of *pvpA* and other surface protein genes will be required to better elucidate relationships among these strains.

A desired application of the seminested PCR-RFLP procedure is to detect and rapidly differentiate *M. gallisepticum* strains present in infection of commercial poultry flocks directly from tracheal samples, thus eliminating the need for culture. Detection of *M. gallisepticum* directly from tracheal scrapings with *pvpA* PCR was demonstrated in samples from commercial layer hens. Probably due to the low tracheal levels of *M gallisepticum* in chronically infected chickens (11), attempts to isolate the organism were not successful and the only evidence of infection was weak plate agglutination reactions. *M. gallisepticum* infection in acutely infected broiler-breeder flocks in Israel was diagnosed by the *pvpA* PCR test, by *M. gallisepticum* PCR, and by isolation. The lower percentage of positive samples in the *pvpA* PCR test, seen in Table 4, suggests that the sensitivity of this test may be less than that of the *M. gallisepticum* PCR test, which is <50 CFU (S. Levisohn, unpublished data). Nonetheless, this does not affect the diagnostic results in these cases, since these are based on the flock status rather than individual samples. However, further optimization of the PCR test may be necessary in order to improve the sensitivity as needed for testing of clinical samples.

Analysis of clinical isolates from Israel by *pvpA* PCR-RFLP showed that all samples have a 400-bp amplicon. In addition, an identical and unique RFLP pattern was observed for three Israel isolates, which had been previously characterized and differentiated by RAPD analysis (data not shown). Our findings suggest that the *pvpA* gene present in Israeli outbreak-

related isolates is more conserved than the *pvpA* gene of U.S. isolates. However, these strains are readily differentiable from the vaccine strains.

The data presented here indicate that the *pvpA* gene of *M. gallisepticum* is a reliable target gene for differentiation of vaccine strains from field isolates based on either size variation, RFLP analysis, or sequencing of PCR products.

Moreover, it was demonstrated that sequence analysis of the *pvpA* gene could be utilized for epidemiology studies of *M. gallisepticum* outbreaks. This is the first study where a surface protein gene of *M. gallisepticum* has been utilized for molecular epidemiological analysis of a significant number of field isolates. Increasing sequence analysis of the *pvpA* and other genes will allow a better understanding on the origin of *M. gallisepticum* outbreaks and therefore will facilitate better strategies to control the disease. In addition, with further optimization, the *pvpA* PCR assay can be utilized as a tool to detect and identify specific *M. gallisepticum* strains directly from clinical material without the need for isolation.

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