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ScienceDirect
Veterinary Microbiology 129 (2008) 139–149
veterinary microbiology
www.elsevier.com/locate/vetmic

A recombinant PvpA protein-based diagnostic prototype for rapid screening of chicken *Mycoplasma gallisepticum* infections

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Received 19 April 2007; received in revised form 18 November 2007; accepted 28 November 2007

Abstract

Mycoplasma gallisepticum is the primary agent of chronic respiratory disease causing important economic losses in the poultry industry. Serological monitoring is essential to maintain mycoplasma-free breeder flocks and often complicated by the cross-reactions between different mycoplasma species. To overcome serological cross-reactions, a large fragment of the *M. gallisepticum* PvpA cytadhesin, species-specific surface-exposed protein, was produced in *E. coli* as a recombinant protein (rPvpA336) and used as a potential diagnostic antigen. The rPvpA336 protein possesses 336 mycoplasma-specific amino acids with relative molecular weight of 44 kDa. A deletion region of 37 amino acids was identified when compared to the wild-type PvpA protein. Immunoreactivity of the rPvpA336 protein has been demonstrated by Western blot analysis with *M. gallisepticum*-positive and -negative chicken sera. Furthermore, an enzymatic rapid immunofiltration assay (ERIFA) prototype based on the rPvpA336 protein has been developed and its species-specific detection capability has been demonstrated by using *M. gallisepticum* and/or *M. synoviae*-positive and -negative chicken sera. In addition to its species-specificity, the ERIFA prototype presents certain advantages such as rapidity, field-applicability and cost-effectiveness. Therefore, these advantages would make the prototype a species-specific rapid diagnostic tool of choice in the field and limited laboratory conditions for screening *M. gallisepticum* infections.

Keywords: *Mycoplasma gallisepticum*; Recombinant PvpA; Serodiagnostic prototype; Chicken

1. Introduction

Mycoplasma gallisepticum is the primary agent of chronic respiratory disease in chickens, and infectious sinusitis in turkeys causing important economic losses in poultry industry. Colonization of the chicken respiratory tract by *M. gallisepticum* is the basis of the disease and mediated by several cytadhesin-related molecules such as MGC1 or GapA (Keeler et al., 1996; Goh et al., 1998), MGC2 (Hnatow et al., 1998), MGC3 or CrmA (Yoshida et al., 2000) and PvpA

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doi:10.1016/j.vetmic.2007.11.028

(Yogev et al., 1994). To maintain mycoplasma-free breeder flocks, the disease is essentially controlled by serological monitoring often complicated by the cross-reactions between different mycoplasma species (Noormohammadi et al., 1998; Dufour-Gesbert et al., 2001; Bencina, 2002; Feberwee et al., 2005). The cross-reactions, due to the presence of identical antigens or antigenic determinants, have been overcome by using recombinant fusion proteins including species-specific domain of *M. synoviae* (Noormohammadi et al., 1999) and *M. meleagridis* (Ben Abdelmoumen Mardassi et al., 2007) as potential diagnostic antigens.

In the case of *M. gallisepticum* infections, the PvpA cytadhesin could be a potential diagnostic antigen of choice since this surface-exposed protein is accessible to the host immune response (Boguslavsky et al., 2000) and its species-specific and immunogenic properties have clearly been demonstrated (Yogev et al., 1994). Considering its frequent phase- and size-variability in expression and among different strains (Yogev et al., 1994; Levisohn et al., 1995; Boguslavsky et al., 2000), a recombinant PvpA protein (rPvpA336) was produced as a standard target antigen and a rapid serodiagnostic prototype based on the rPvpA336 protein was developed for screening *M. gallisepticum* infections in chicken. The immunoreactivity and specificity of the rPvpA336 protein as well as the potential use of the species-specific diagnostic prototype were discussed.

2. Materials and methods

2.1. Bacterial strains and growth conditions, antibody and chicken sera

Genomic DNA and bacterial lysate were obtained from *M. gallisepticum* strain Pendik provided from Pendik Veterinary Control and Research Institute (İstanbul, Turkey). *E. coli* DH5α (Takara Inc., Paris, France) was grown at 37 °C in LB (Luria-Bertani) broth. In the case of the transformed *E. coli* DH5α strain, LB broth contained 50 μg/ml of ampicillin.

Mouse monoclonal anti-HisTag antibody (His-probe (H3): sc-8036) purchased from Santa-Cruz Biotechnology Inc. (California, USA) was used as positive control of the recombinant protein in Western blot analysis. Chicken sera were obtained from a private diagnostic laboratory Protekt (İstanbul, Turkey) regularly monitoring *M. gallisepticum* infection by using RSA test (rapid serum agglutination) and ELISA. RSA test antigens were obtained from Intervet-Turkey (Nobilis MG antigen, İstanbul, Turkey) and Pendik Veterinary Control and Research Institute. ELISA kits were purchased from BioChek (AN Gouda, Holland) and IDEXX Laboratories, Inc. (Maine, USA). The chicken sera were tested or retested with ELISA kits (BioChek) according to the manufacturers' instructions for detecting *M. synoviae* or *M. gallisepticum* antibodies.

2.2. PCR amplification and cloning of the pvpA gene into pCold I vector

pColdI expression vector is used for obtaining recombinant polyhistidine (HisTag) fusion proteins (Takara Inc.). pColdI expression vector contains cold shock protein (cspA) promoter region allowing the induction of the expression of recombinant proteins with IPTG (isopropyl-β-D-thiogalactopyranoside) at 15 °C-growth conditions.

M. gallisepticum genomic DNA was purified by "Genomic DNA Purification Kit" (Bio Basic Inc., Ontario, Canada) from inactivated bacteria following the instructions of the manufacturer. For PCR amplification, forward and reverse primers were synthesized by Bio Basic Inc. Forward primer (5′ GGGCAAGAGCTCAATAAATTAAAAAAAC 3′) contains SacI and reverse primer (5′ GCTC-GGAATTCCCCACCTTATGGTCTTGG 3′) contains EcoRI restriction enzyme sites. PCR amplification was performed in a 50 μl reaction mixture containing 1.5 mM of $MgCl_2$, 10× PCR buffer, 1 mM of each dNTP, 30 pmol of each primer, 2 U/μl of Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania), and 200 ng of genomic template DNA, in a DNA thermal cycler. The initial cycle of 5 min of denaturation at 94 °C was followed by 35 cycles of 30 s of denaturation at 94 °C, 30 s of annealing of the primers at 58 °C, 1 min 40 s of extension at 72 °C, with a final 10 min extension step at 72 °C. The *pvpA336* amplicon was purified by QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) and digested by *Eco*RI and *Sac*I (Fermentas UAB). Cohesive-end ligation of the restricted amplicon into pColdI vector was performed

with the aid of T4 DNA ligase (Fermentas UAB). The resulting plasmid was designated as pCold-*pvpA336*.

2.3. DNA sequencing

DNA sequencing was performed with "ABI 310 Capillary DNA Sequencer" (Global Medical Instrumentation Inc., Minnesota, USA) and "ABI PRISM BigDye Terminator Cycle Sequencing Kit" (Applied Biosystems, California, USA). Initially, *pvpA336* gene fragment was amplified by PCR from pCold-*pvpA336* as template by using forward (5′ GCACGCCATATCGCCGAAAGG 3′) and reverse (5′ CCAAATGGCAGGGATCTTAGATTCTGTGC 3′) primers and purified. The sequencing reaction solution with a volume of 20 μl consisted of 8 μl of Big Dye Mix (4 μl of mix and 4 μl of 5× sequencing buffer), 5–20 ng of template, 2 μl of primer and dH_2O. The DNAs were amplified for 25 cycles (10 s at 96 °C, 5 s at 55 °C and 4 min at 60 °C). Bases not participating in polymerization were removed from the reaction solution by "Dye-Ex Kit".

2.4. Nucleotide and amino acid sequence data

The nucleotide sequence of the *pvpA336* gene and deduced amino acid sequence of the rPvpA336 protein obtained from *M. gallisepticum* strain Pendik have been deposited in NCBI-GenBank database under the accession nos. DQ989519.2 and ABJ96344, respectively. For sequence similarity and homology searching, the Blast program at the NCBI web server was used.

2.5. Transformation and expression of recombinant rPvpA336 protein in E. coli

Competent *E. coli* DH5α cells were transformed with the pCold-*pvpA336* vector and ampicillin-resistant transformants were selected in LB broth as previously described (Sambrook et al., 1989). Single colony of the transformant was cultured and pCold-*pvpA336* vectors were purified with Mini-prep DNA Extraction Kit (Qiagen GmbH) and digested with EcoRI and SacI enzymes to confirm the presence of *pvpA336* insert. Then, the transformants were cultured until they reached 0.3–0.4 absorbance at 600 nm. Expression of the recombinant PvpA336 protein was induced by the addition of IPTG (Fermentas UAB) at a final concentration of 0.5 mM to the culture. The incubation was continued at 15 °C for 24 h with shaking at 200 rpm/min. The cells were collected by centrifugation at 4000 × *g* for 20 min and washed three times in 0.15 M NaCl and resuspended in lysis buffer (125 mM Tris–HCl pH 6.8, 1% SDS). Bacteria were boiled for 10 min and then centrifuged at 20,000 × *g* for 20 min to discard the cell debris.

2.6. SDS-PAGE and Western blot analysis

Whole cell extracts of *M. gallisepticum* and the recombinant *E. coli* as well as the purified rPvpA336 protein were separated on 10% polyacrylamide gels (Laemmli, 1970) and stained with coomassie brilliant blue or electro-transferred to polyvinyldifluoride (PVDF) membranes (0.2 μm pore size, Immobilon-P, Sigma–Aldrich, St. Louis, USA) for 1 h at 0.8 mA/cm^2 using a semi-dry transblotter (Towbin et al., 1979). The membrane strips blocked with 1% gelatin from cold water fish skin (Sigma–Aldrich) in PBST (PBST/FG, pH 7.4; 0.1% Tween 20) were incubated with either chicken sera at a dilution of 1:200 for 1 h or anti-HisTag Mouse monoclonal antibody (IgG_1, Santa-Cruz) at a dilution of 1:200 for 90 min at room temperature with gently shaking. The membranes were then incubated with either AP conjugated Rabbit anti-chicken IgY (Sigma–Aldrich) or AP conjugated goat anti-mouse γ chain specific (Sigma–Aldrich) antibody solutions for 1 h at room temperature with shaking. Color reaction was developed with the addition of BCIP/NBT-blue liquid substrate system for membranes (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, Sigma–Aldrich) for 10 min and stopped by washing with distilled water.

2.7. Purification of the recombinant rPvpA336 protein

Whole cell extract of the induced bacteria dialyzed against PTU buffer (phosphate–Tris–urea buffer: 0.1 M NaH_2PO_4, 10 mM Tris, 8 M urea pH 8) was mixed with 1 ml of Ni-NTA agarose beads (Ni-nitrilotriacetic acid, Qiagen) by gently shaking at 200 rpm at room temperature for 1 h and loaded to the empty column and washed with PTU buffer pH 6.3. Two-step elution of the recombinant rPvpA336 protein was performed with PTU buffer at pH 5.9 and 4.5. The fractions containing rPvpA336 protein were determined by SDS-

PAGE. The protein band corresponding to rPvpA336 protein was excised from the polyacrylamide gel and incubated in the elution buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1 mM EDTA pH 7.5) with shaking. The rPvpA336 diffused into the elution buffer was analyzed by SDS-PAGE and then, highly purified recombinant rPvpA336 protein was used for Western blot and enzymatic rapid immunofiltration assays.

2.8. Enzymatic rapid immuno-filtration assays (ERIFA)

Enzymatic rapid immuno-filtration assay (ERIFA) is a version of ELISA, except that it is performed on a nitro-cellulose (NC) membrane (Schleicher&Schuell, Germany) included in an individual plastic cassette and the reaction time is less than 10 min. NC membrane is placed onto a high capacity absorbent pad (Schleicher&Schuell) for capturing all reagents and solutions used in the assay. For detecting anti-PvpA antibodies, 0.5 μl of highly purified rPvpA336 protein (1 μg/μl) as target antigen and 0.2 μl of chicken sera diluted at 1:5 as internal control of the test were manually adsorbed onto NC membrane. After wetting, NC membrane was saturated with 100 μl of 1% cold-water fish gelatin (PBST/FG) that was also used as washing and diluting solutions for all samples and detection reagents. After saturation of NC membrane, 50 μl of serum samples were added: samples were flowed through the membrane, then washed with 50 μl of PBST/FG. Fifty microliters of AP conjugated anti-chicken IgY solution (Sigma–Aldrich) were added and flowed through NC membrane. The membrane was washed four times and 50 μl of BCIP/NBT substrate solution (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, Sigma–Aldrich) was added. Color development was stopped in 2 min maximum by washing solution. The results were estimated following their intensity and visualization time.

3. Results

3.1. Cloning and DNA sequencing of the pvpA gene and its amino acid sequence

DNA sequencing analysis of the *pvpA* gene fragment integrated into pColdI vector demonstrated that it was composed of 1008 bp encoding 336 amino acids of *M. gallisepticum* PvpA cytadhesin. Based on these results, the *pvpA* gene fragment insert was named as *pvpA*336 and the corresponding recombinant protein as rPvpA336.

In Fig. 1, a homology comparison was given with the wild-type putative variable cytadhesin protein PvpA of *M. gallisepticum* R strain (GenBank accession no.: AAF67108). A noteworthy finding of the homology comparison is that the rPvpA336 protein contains a deletion region of 37 amino acids from positions 264 to 300 amino acid residues according to the wild-type R strain without deletion. This deletion area is distributed from the last 11 amino acids of DR1 (direct repeated region, 264–275 aa residues) to the beginning of DR2 region (301–352 aa residues). In addition, the rPvpA336 protein also possesses different amino acid residues at positions 16, 116, 124, 169, 173,174, 177, 187, 193, 209, 247, 248, 275, 287 and 288 (Fig. 1). The rPvpA336 amino acid residues at positions 275, 287 and 288 correspond to positions 311, 323 and 324 of the wild-type PvpA protein. A codon insertion into the *pvpA336* gene, which encodes an additional amino acid residue at the position 165, was also detected. Taken together, the rPvpA336 protein presents 85% homology with the wild-type PvpA protein.

3.2. Expression and purification of the rPvpA336 protein

Fig. 2 shows SDS-PAGE analysis of whole-cell extract of non-induced *E. coli* (lane 1) harboring recombinant expression vector pCold-*pvpA336* in comparison with those of the IPTG-induced *E. coli* (lanes 2–3). High-level expression of the recombinant rPvpA336 protein of approximately 44 kDa was detected in the IPTG-induced bacterial cell extract, while non-induced bacteria did not express the recombinant protein. Purification of rPvpA336 protein was performed following confirmation by Western blot analysis with monoclonal anti-HisTag antibody. The rPvpA336 protein was primarily purified by Ni-NTA affinity chromatography and analyzed by SDS-PAGE. To remove slight contaminants of *E. coli*, a further purification was performed by SDS-PAGE separation followed by elution of the rPvpA336 protein from the polyacrylamide gel in TNE elution

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  1 MGQELNKLKKHKIISIVLMAIGALILLSGIALTAVIASPINSVEVTEMMN
       ELNKLKKHKIISMVLMAIGALILLSGIALTAVIASPINSVEVTEMMN

 51 GQEVTTTKKISTFAFLINMLPNYQLSTLGYLQITGAAAGLVVGIVLLALG
    GQEVTTTKKISTFAFLINMLPNYQLSTLGYLQITGAAAGLVVGIVLLALG

101 ATFFVKTRRKTNEMLAALQDAEEEEEVAQEEQAEENVEATPTQQAEVKTE
    ATFFVKTRRKTNEMLTALQDAEEVEEVAQEEQAEENVEATPTQQAEVKTE

151 QLIGTQLVTTDVAS-TQAVGTEEVQGVLLPPSQQPTEMRPAPSPMGSPKL
    QLIGTQLVTTDVASTTQAAGTEKAQGDLLPPSQQPTGMRPAPLPMGSPKL

200 LGPNQAGHPQHGPRPMNAHPGQPRPQQAGPRPMGAGGSNQPRPMPNGLQN
    LGPNQAGHSQHGPRPMNAHPGQPRPQQAGPRPMGAGGSNQPRPMPNRPQN

250 PQGPRPMNPQGDPRPQPAGVRPNSPQNSQPRPMPNKPQGPRPMGAPNPQP
    PQGPRPMNPQGDPR-----------------------------------

300 GPQQAGPRPMGVGGSNQPRPMPNGLQNPQGPRPMNPQGDPRPQPAGVRPN
    -PQQAGPRPMGAGGSNQPRPMPNRPQNPQGPRPMNPQGDPRPQPAGVRPN

350 SPQANQPGRRPTPNNPQGPRPMGPRPNGGPNRA 382    (AAF67108)
    SPQANQPGRRPTPNNPQGPRPMGPRP             (rPvpA336)
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Fig. 1. Homology comparison of the wild-type PvpA protein (upper lanes marked with amino acid number, AAF67108 indicates GenBank accession number) with the rPvpA336 (lower lanes, GenBank accession no.: ABJ96344). Bold letters underlined indicate different amino acid residues between the wild-type PvpA and rPvpA336 proteins and the dashes from 264 to 300 amino acid residues represent the deletion region of the rPvpA336.

buffer. In this way, very highly purified rPvpA336 protein has been obtained (Fig. 2, lane 4) and its molecular weight was estimated as 44 kDa in comparison with protein molecular weight marker (Fig. 2).

3.3. Immunoreactivity of the rPvpA336 protein

To determine immunoreactivity of the rPvpA336 protein, *M. gallisepticum*-positive and -negative chicken sera previously evaluated with RSA or ELISA were analyzed and confirmed with Western blot assay by using whole-cell extract of *M. gallisepticum* Pendik strain as antigen. The results showed that positive chicken sera recognized several bacterial antigens of different molecular weight, but negative sera did not react with any antigens (data not shown). Then, chicken sera were individually classified as *M. gallisepticum*-positive or -negative confirmed sera and used for further experiments. In order to determine the exact location of the rPvpA336 protein, a monoclonal anti-HisTag antibody recognizing six histidine residues was used in Western blot analysis

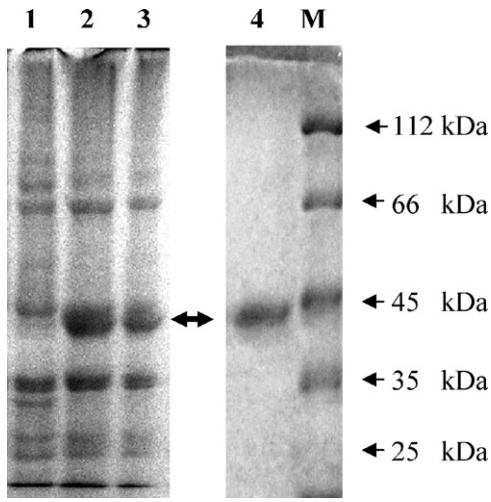

Fig. 2. SDS-PAGE analysis of the recombinant rPvpA336 protein expressed in *E. coli*. Lane 1, whole-cell extract from non-induced bacteria. Lanes 2 and 3 represent 20 and 10 μl samples of whole-cell extract from IPTG-induced bacteria, respectively. Lane 4, purified rPvpA336 protein. The position of rPvpA336 proteins is indicated by two-headed arrow. M: molecular weight marker indicated in kDa.

with whole-cell extract of *E. coli* cells expressing the recombinant protein and its purified form.

Typical results of Western blot analysis performed with whole-cell proteins of the IPTG-induced *E. coli* and affinity purified rPvpA336 protein were shown in Fig. 3A and B, respectively. Monoclonal anti-HisTag antibody strongly reacted with a protein band of 44 kDa corresponding to the recombinant protein (Fig. 3A and B, lane 1). All of the *M. gallisepticum*-positive and -negative chicken sera recognized several protein bands of *E. coli* cells expressing the recombinant protein (Fig. 3A, lanes 2–8). In contrast, only positive chicken sera reacted with 44 kDa protein band that was also recognized by monoclonal anti-HisTag antibody (Fig. 3A, lanes 4–8). These results were confirmed with the purified rPvpA336 protein-based Western blot assays (Fig. 3B). All of the positive sera (Fig. 3B, lanes 4–8) and monoclonal anti-HisTag antibody (Fig. 3B, lane 1) reacted less or more intensely with the purified recombinant protein band of 44 kDa, while no reaction was observed with negative sera (Fig. 3B, lanes 2 and 3). These results showed that the recombinant rPvpA336 protein still preserves its immunoreactivity in the purified and non-purified forms and can be used as a diagnostic antigen.

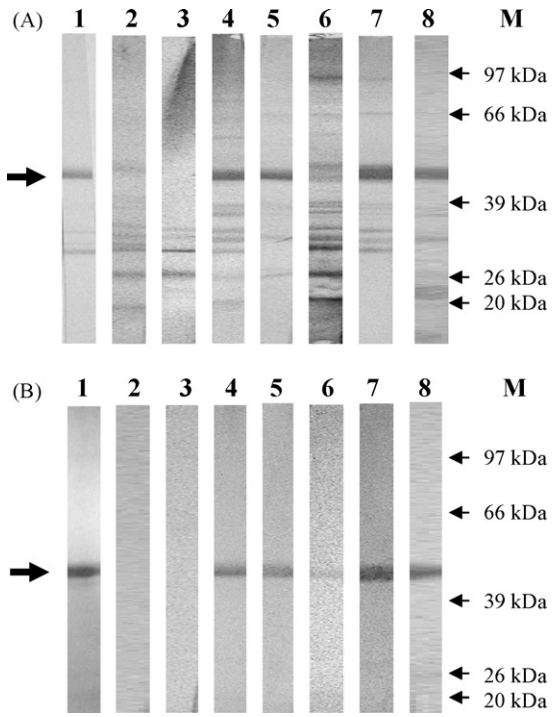

Fig. 3. Western blot analysis using *M. gallisepticum*-positive (lanes 4–8) and -negative chicken sera (lanes 2 and 3) with whole-cell extract from IPTG-induced *E. coli* (A) and purified rPvpA336 protein (B). Lanes 1A and 1B, immunostaining with monoclonal anti-HisTag antibody. Large arrows show the position of rPvpA336 protein band. M: molecular weight marker indicated in kDa.

3.4. Development of the rPvpA336-based ERIFA test prototype

To screen anti-PvpA antibody in *M. gallisepticum* (MG)-infected chicken sera in the field and limited laboratory conditions, an enzymatic rapid immunofiltration assay prototype (ERIFA) has been developed by using rPvpA336-positive or -negative sera confirmed with Western blot analysis. Diluted chicken sera as internal control of the test were dotted onto NC membrane at the left side of the cassette (C, control) and the recombinant rPvpA336 protein as target antigen was adsorbed at the right side of the cassette (T, test). Development and optimization of the ERIFA prototype was done with nine confirmed rPvpA336-positive or -negative sera numbered from 1 to 9 (Fig. 4). Positive reaction of the internal control (dashed arrow) in all tests indicates that the tests work correctly. As shown in Fig. 4, rPvpA336-negative chicken sera (samples 4 and 5) have also been found negative whereas all positive sera have recognized less or more intensely the rPvpA336 protein (full arrows). Test results can be completed and evaluated in less than 10 min. These results demonstrated that this diagnostic prototype was very rapid and capable of detecting the presence of anti-rPvpA336 antibodies from MG-positive chicken sera.

3.5. Specificity of the rPvpA336-based ERIFA prototype

Chicken sera evaluated with ELISA (Bio Check) as MG and/or *Mycoplasma synoviae* (MS)-negative or -positive were tested with the rPvpA336-based ERIFA prototype in order to determine its species-specificity. Five MG and MS-negative sera (samples 1–5), 10 MG-positive and MS-negative sera (samples 6–15), 7 MG-negative and MS-positive sera (samples 16–20, 23 and 24), 2 MG and MS-positive sera (samples 21 and 22) and mouse monoclonal anti-HisTag antibody (sample 25) were evaluated with ERIFA prototype

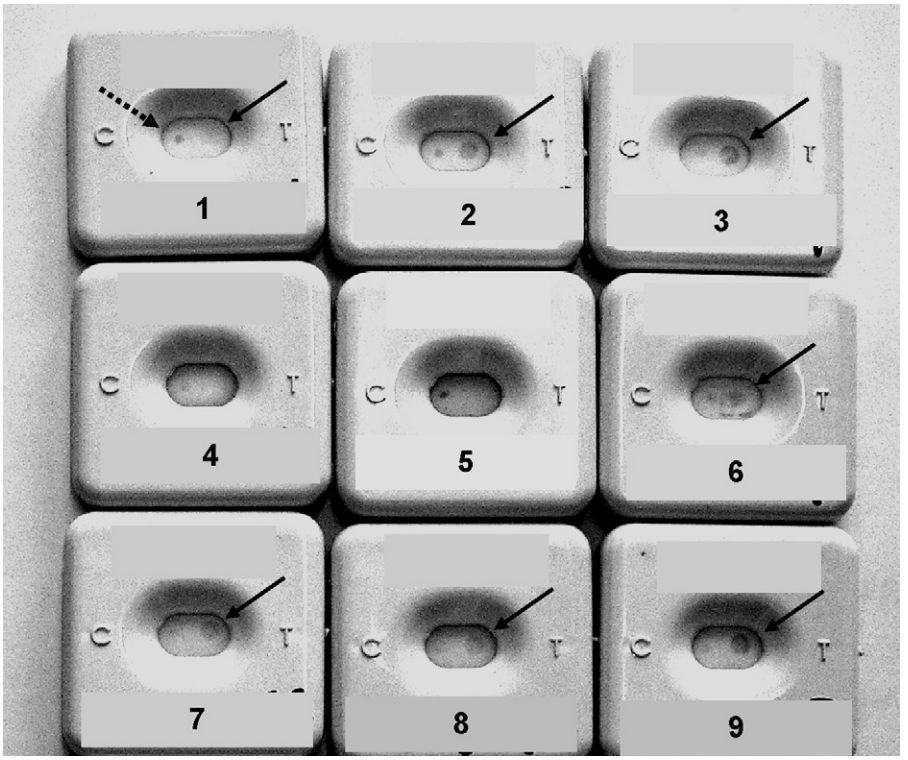

Fig. 4. Typical ERIFA prototype results with *M. gallisepticum*-positive (nos.: 1–3 and 6–9) and -negative (nos.: 4 and 5) chicken sera. Arrows indicate internal control of the test on the left side and the recognition of the rPvpA336 on the right side.

(Fig. 5). As shown in Fig. 5, internal control of the test samples from 1 to 24 was found reactive, except the sample 25 where anti-mouse IgG conjugate instead of anti-chicken IgY conjugate was used in this test for demonstrating the presence of the rPvpA336 protein. No reaction was observed with 5 MG and MS-negative sera (samples 1–5) and 7 MG-negative and MS-positive sera (samples 16–20, 23 and 24) whereas 10 MG-positive and MS-negative sera (samples 6–15) and 2 MG and MS-positive sera (samples 21 and 22) have reacted less or more intensely with the rPvpA336 protein (full arrows in Fig. 5). Positive reaction intensity of chicken sera with the rPvpA336 protein was found uncorrelated with their MG–ELISA titer. For example, the sample 15 with low response in MG–ELISA reacted as intense as the sample 14 having high response in MG–ELISA. These results demonstrate that the rPvpA336-based ERIFA test allows rapid and species-specific screening of sera from *M. gallisepticum*-infected chickens.

4. Discussion

Immunofiltration assay, also called as ‘‘flow-through assay’’, is a well-known method and various tests have been developed and widely used as sensitive diagnostic tools in human medicine (Xiao et al., 2003; Foglia et al., 2004; Daniel et al., 2005; O’Connell et al., 2006). Though being easily applicable to the diagnosis purposes and adaptable to the field and limited laboratory conditions, the use of this immunoassay method is uncommon in veterinary medicine (Abdel-Hamid et al., 1999; Yamazaki et al., 2004). In this study, an enzymatic rapid immunofiltration assay (ERIFA) prototype was developed to screen *M. gallisepticum*-infected chickens by using the purified recombinant PvpA protein known as a species-specific antigen (Yogev et al., 1994; Boguslavsky et al., 2000). For this purpose, a large fragment of *pvpA* gene composed of 1008 nucleotides encoding a polypeptide of 336 amino acid residues (rPvpA336)

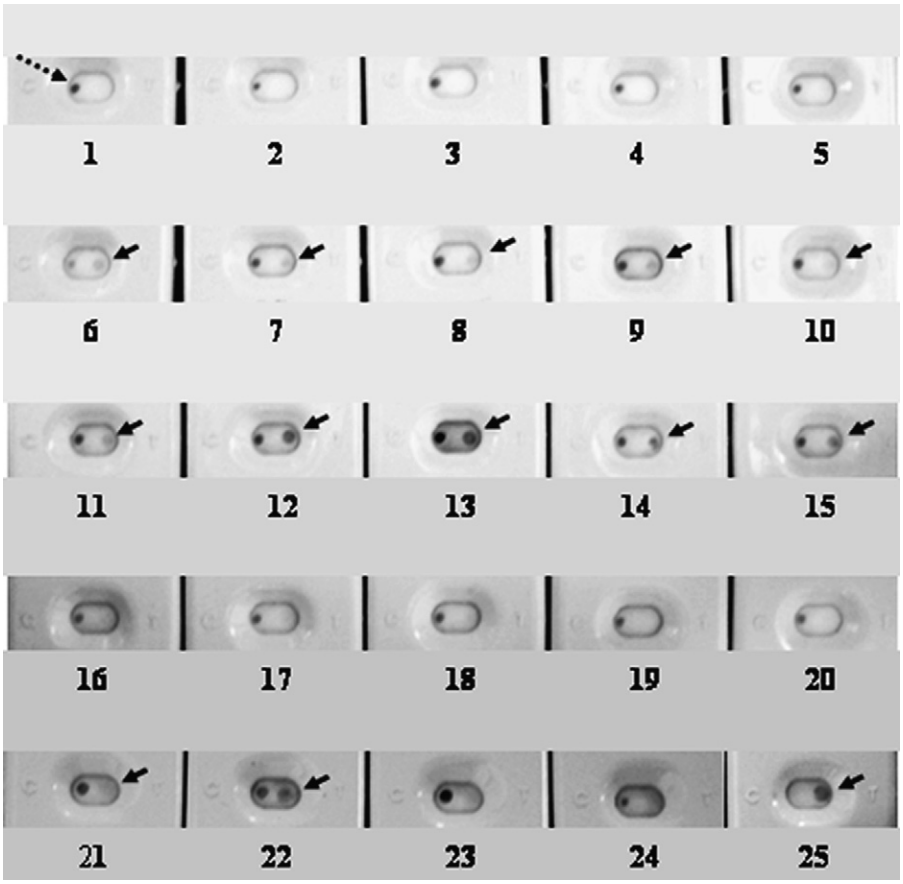

Fig. 5. Specificity of the rPvpA336-based ERIFA test by using *M. gallisepticum* (MG) and *M. synoviae* (MS)-negative sera (samples 1–5), MG-positive and MS-negative sera (samples 6–15), MG-negative and MS-positive sera (samples 16–20, 23 and 24), MG and MS-positive sera (samples 21 and 22) and mouse monoclonal anti-HisTag antibody (sample 25). Arrows indicate internal control of the test on the left side and the recognition of the rPvpA336 on the right side.

was cloned into pColdI vector, expressed in *E. coli* and affinity purified. Homology comparison of the recombinant rPvpA336 protein with the wild-type PvpA protein (GenBank accession no.: AAF67108) revealed the presence of a deletion region of 37 amino acids taking place at the proline-rich C-terminal region from the last 11 amino acids (residues 264–275) of the direct repeat region DR1 to the beginning of DR2 region (Fig. 1). This deletion was localized within the hot-spot deletion domain of the *pvpA* gene as previously described for various *M. gallisepticum* strains (Boguslavsky et al., 2000; Liu et al., 2001) and as its result, relative molecular weight of the rPvpA336 protein was found as 44 kDa in SDS-PAGE analysis (Fig. 2). In spite of the deletion region and divergence in 15 amino acid residues, the recombinant rPvpA336 protein presents very high level of homology with the wild-type PvpA protein.

Immunostaining of a 44 kDa protein band in crude and purified forms (Fig. 3A and B) with monoclonal anti-HisTag antibody confirmed the expression and location of the recombinant rPvpA336 protein derived from MG. Recognition of the 44 kDa protein band in the same Western blot assays by the chicken sera confirmed as MG-positive, but not with negative sera strongly suggests that the recombinant rPvpA336 protein still preserves its antigenic properties while it contains a deletion region of 37 amino acids. This result is not surprising because the reactivity of several native PvpA proteins containing various deletions

have also been demonstrated with Western blot analysis (Boguslavsky et al., 2000). Primary evidence of the immunogenicity of PvpA protein has been demonstrated by Western blot analysis with a serum pool of infected chickens recognizing the bacterial surface antigens obtained only from PvpA producing clonal variants of *M. gallisepticum* R strain, but not from PvpA-negative clones (Yogev et al., 1994). Besides, the immunoreactivity of the recombinant PvpA protein has also been shown with *E. coli* bacterial (Boguslavsky et al., 2000) and Avipox viral recombinant expression systems (Saitoh et al., 1999). Likely, its highly hydrophilic outer-membrane domain within 100–382 amino acid residues of the wild-type molecule (Boguslavsky et al., 2000) may contain several immunodominant domains recognized by the natural host immune response, but they have not yet been determined. In this respect, our preliminary results (data not shown) indicate that their location could essentially be present exterior to the direct repeat regions DR1 and DR2, probably within the segments encompassing 110–220 and 350–382 amino acid residues of the wild-type PvpA protein.

From the results of this study and those previously described for PvpA (Yogev et al., 1994; Saitoh et al., 1999; Boguslavsky et al., 2000), the recombinant rPvpA336 protein can be considered as a highly immunoreactive species-specific molecule. Therefore, it was used as a target antigen in the development of a diagnostic prototype to avoid inter-species cross-reactivity frequently observed with the whole-cell, their extracts or surface molecules harboring identical antigenic determinants (Noormohammadi et al., 1998; Dufour-Gesbert et al., 2001; Bencina, 2002; Feberwee et al., 2005). Cross-reactions between the sera from chicken infected with *M. gallisepticum* and *M. synoviae*, two major avian mycoplasmosis agents sharing common antigenic determinants within their respective pMGA1.7 and VlhA surface proteins, have been overcome by an immunoassay based on the recombinant fusion protein including a species-specific domain of MSPB encoded by *vlhA* gene of *M. synoviae* (Noormohammadi et al., 1999). In addition, use of recombinant proteins mimicking species-specific antigens or antigenic determinants in the development of diagnostic assays has several advantages over those obtained from cultivated bacteria (Noormohammadi et al., 1999; Okada et al., 2005; Ben Abdelmoumen Mardassi et al., 2007).

In our knowledge, no species-specific recombinant antigen-based immunoassay models adapted to the field and limited laboratory conditions for screening *M. gallisepticum*-infected chickens was described in the literature. Considering species-specific and immunogenic properties of the PvpA protein (Yogev et al., 1994; Levisohn et al., 1995; Boguslavsky et al., 2000), usefulness of the rPvpA336 protein as a standardized species-specific antigen has been demonstrated not only by Western blot analysis but also by ERIFA test (Figs. 3–5). In fact, with the ERIFA test, only MG-positive and MS-negative or MG- and MS-positive chicken sera were found reactive but not MG- and MS-negative or MG-negative and MS-positive sera (Fig. 5). Despite a limited number of chicken sera were tested to evaluate the ERIFA prototype, these preliminary results indicate that the rPvpA336-based ERIFA test is able to avoid the false positive reactions for the screening of MG-positive sera. In contrast, the false negative reactions with the rPvpA336-based ERIFA test may not be excluded since they could be due to the infections with some MG strains lacking PvpA protein (Bencina et al., 2003). To overcome the possibility of non-detection of the infections due to MG strains lacking PvpA protein, the ERIFA test could therefore be enriched with a second highly conserved and immunogenic antigen of *M. gallisepticum* or its species-specific antigenic determinants. Although MG infections caused by PvpA lacked strains are extremely rare, antigenic variability due to the size- and phase-variation of the PvpA protein among MG strains is more frequent (Levisohn et al., 1995; Boguslavsky et al., 2000). As the PvpA proteins from various MG strains contain a number of common immunodominant epitopes, some MG-infected chickens from the same flock could likely develop detectable antibody response to the PvpA variant proteins including the truncated ones.

In conclusion, the adaptability of the enzymatic rapid immunofiltration assay to *M. gallisepticum* diagnosis has been shown through the detection of anti-PvpA antibody with ERIFA diagnostic prototype. This immunoassay prototype can already be considered as a promising field-diagnostic tool since its application is no longer than 10 min without any specific equipment and the results are evaluated as

positive or negative in function of the intensity and appearance time of the color reaction. Specificity and high sensitivity of the assay are assured by the species-specific rPvpA336 protein and the enzymatic reaction. Taking these advantages into account, such a rapid, cost-effective and species-specific diagnostic tool has the potential of being included in the panel of *M. gallisepticum* screening tests in the field and limited laboratory conditions. Nevertheless, its potential as a field test needs to be validated with more sera from chickens experimentally and naturally infected by *M. gallisepticum*, *M. synoviae* and *M. meleagridis*. This investigation is currently undergoing in our laboratory.

Acknowledgment

This study was supported by Grant no. 104T271 from The Scientific and Technical Research Council of Turkey (TÜBİTAK, Turkey).

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