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ABSTRACT

Mycoplasma gallisepticum (MG) is a member of the most important avian mycoplasmas, causing chronic respiratory disease in chickens and lead ing to important economic losses in the poultry in dustry. Recombinant technology represents a strategic approach used to achieve highly reliable and specific diagnostic tests in veterinary diseases control: in par ticular this aspect is crucial for confirming mycoplasma infection and for maintaining mycoplasma-free breeder flocks. In this study, we identified a component of the pyruvate dehydrogenase dihydrolipoamide acetyltrans ferase (i.e., E2) protein by 2-dimensional electrophore sis (2-DE), characterized it in immunoblotting assays, and analyzed its recombinant (r-E2) in a rec-ELISA test. For full-length protein expression in Escherichia coli (EC) a point mutation was introduced. A rab bit antiserum produced against r-E2 was tested in a Western Blot using different samples of Mycoplasma species. The results showed the applicability of site directed mutagenesis, with a good yield of the r-E2 after purification. Also, anti-E2 serum reacted with all the tested MG strains showing no cross reaction with other mycoplasmas. The developed E2 ELISA test was capable of detecting MG antibodies in the sera examined. Those results demonstrate the anti genic stability of the E2 protein which could repre sent a recombinant antigen with potential diagnostic applications.

Key words: Mycoplasma gallisepticum, recombinant protein, E2

INTRODUCTION

Mycoplasma gallisepticum (MG) is an important pathogen of poultry worldwide that represents consid erable economic losses to the poultry industry, causing chronic respiratory disease in chickens and turkeys, re duction in egg production and quality, poor hatchability and feed efficiency, and increase in mortality and car cass condemnations besides the medication costs. (Ley et al., 2009; Ghorashi et al., 2010).

The genus Mycoplasma (class Mollicutes) has evolved by degenerative or reductive evolution, accompanied by significant loss of genomic sequences and progressive adaptation to their specific hosts. They are distinguish able from other bacteria by their minute size and lack of a cell wall, and because of that, the surface proteins (lipoproteins) anchored or embedded in the cell mem brane play a crucial role in the interactions between the mycoplasmas and their hosts (Benˇcina et al., 1994). In the absence of a cell wall, the surface of the my coplasma membrane constitutes a critical interface in the infectious process, mediating basic functions such as the transport of nutrients as well as more complex interactions with the host cells and the host immune defenses.

The chronic nature of Mycoplasma infections demon strates a failure of the host immune system to deal effectively with these organisms. The well-documented antigenic variation of surface proteins is a major con tributor to the complexity in implementing efficient preventive control measures and accurate diagnostic tests. It also allows MG to evade the host's immune re sponse through the generation of escape variants (Glew et al., 2000; Hudson et al., 2006; Citti et al., 2010) and may contribute to resistance versus antimicrobial therapy by intracellular invasion and survival within eukaryotic cells (Winner et al., 2000).

The knowledge derived from studies of MG proteins undoubtedly has considerable potential for diagnos tic application. Since serological monitoring of poul try is used extensively as a first-line diagnostic test and will continue to be performed on a large scale in the future, there will be an increasing need for well-defined and characterized antigens to enable the development of efficacious and cost-effective control measures(Benˇcinaetal.,1994).Thecurrentlyused tests, such as the commercial rapid serum agglutina tion (RSA) and ELISA, as well as hemagglutination inhibition (HI), have greatly contributed to the control of Mycoplasma infections, but previous work reported a lack of specificity, mainly due to the intrinsic ability of various Mycoplasma species to cross-react (Bu¨yu¨ktanir et al., 2010).

As a consequence, recombinant technology could sig nificantly help to alleviate drawbacks, allowing the pro duction of unlimited amounts of multiple and more specific antigens (Ben Abdelmoumen Mardassi et al., 2008) although identification of immunogenic antigens and genetic manipulations of mycoplasma in general are more laborious than any other prokaryotic genome as far as to successfully express MG proteins in heterolo gous systems such as Escherichia coli (EC), the TGA codons that encode tryptophan in MG genes and, at the same time, are used in EC as a stop codons, need to be replaced by the TGG codon, unless the protein would be produced in a truncated form (Rosati et al., 2000).

Numerous studies have focused on immunogenic pro teins of MG, particularly those involved in hemagglu tination, haemadsorption, and cytadhesion (Milosevic Berlic et al., 2000). Among specific antigens charac terized so far, researchers have identified a large cell surface-exposed lipoprotein (i.e., pMGA) involved in haemagglutination and also pvpA, a putative variable adhesin. Both proteins present polymorphisms and size variation in different MG strains, as well as a phase variable expression (Markham et al., 1992; Boguslavsky et al., 2000; Liu et al., 2001). Considering the limited size of Mycoplasma genomes, the number of genes in volved in generating antigenic diversity on their cell sur face is surprisingly large (Razin et al., 1998).

In previous works, a surface protein called P52 was described and found to be similar to the dihy drolipoamide acetyltransferase (i.e., E2) from several mollicutes (Jan et al., 1996, 2001). This protein is a membrane-associated component of the pyruvate dehy drogenase complex which has been shown to be required for MG in vivo growth and survival in the host. Also, antibodies directed against P52 induced growth inhi bition of cells and activation of the complement sys tem. Partial characterization of P52 protein was limited to amino terminal peptide sequencing and a full anti genic characterization may be required to evaluate its diagnostic potential. The availability of the full genome sequence of MG allows the application of an immune proteome-based strategy to identify potential antigenic sites, useful to further improve current diagnostic tests.

The aim of the present study was to detect, iden tify, and characterize novel MG immunogenic proteins. For that, the proteome of MG was resolved on 2 dimensional electrophoresis (2-DE), blotted on mem brane, and probed against monospecific sera. Along with the known haemagglutinin precursor, a second im munostained protein was identified by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry as E2 pro tein. The corresponding gene was amplified, mutage nized, and cloned in a prokaryotic expression vector. Preliminary antigenic characterization

of recombinant E2 (r-E2) suggests that this protein is well-conserved among and specific within MG isolates, and may be considered for the development of a specific diagnostic assay.

MATERIALS AND METHODS

Two-Dimensional Gel Electrophoresis of Fractionated Mycoplasma Cell Proteins

Mycoplasma gallisepticum American Type Culture Collection (ATCC) Strain 15302, cultured according to Kleven et al. (1998) was centrifuged at 6,000 rpm for 10min at 4◦C and the pellet was washed in a 1X phosphate-buffered saline (PBS) solution 3 times as described (Regula et al., 2000). One milliliter preheated sodium dodecyl sulfate (SDS) 1.0% and 10 µL phenyl methylsulfonyl fluoride (PMSF), 0.1M, were added to the bacterial pellet and boiled for 3min. The sample was sonicated 3 times in ice and centrifuged at 10,000 rpm for 10min. Supernatant was collected and the protein concentration was determined by using a DC protein assay quantification kit (Biorad, Hercules, CA) and purified using the 2D clean up kit (GE Healthcare, Little Chalfont, UK).

The sample was resuspended in a lysis buffer containing 8 M urea,2 Mthiourea, 2.5% 3-[(3-cholamidopropyl)dimethylammonio]-1 propanesulfonate (CHAPS), 1.0% IPG buffer and 20 mMdithiothreitol(DTT,GEHealthcare,Little Chalfont, UK). Strips over the pH range of 4 to 7 (11 cm, Biorad) were subjected to isoelectricfocusing (IEF) using the following parameters: rehydration at 50V for 12h; running at 500V for 1h, 1,000V for 1h, 8,000V for 1h, 1,000 to 8,000V for 4h, and 500 V for 2 h. After isoelectric focusing the gel strips were equilibrated 2× in a buffer containing 7 M urea, 500mM Tris-HCl pH 6.8, 2% SDS, and 10% glycerol. In the first equilibration step 2% DTT was added, and in the second 2.5% iodoacetamide, and incubation was performed with 15 min each. Immediately following equilibration, the IEF strips were transferred onto a 10% SDS-polyacrylamide gel. Electrophoresis was carried out at 170 V for 1 h.

All 2D gel electrophoresis experiments were run in triplicate. On 2 gels, 200 µg proteins were loaded and visualized by Comassie Blue staining; the third gel was used to run 50 µg proteins and visualized by silver stain ing. All gel stains were made with MALDI-compatible colorants. Proteins from one of the Comassie gels were elec tro transferred to nitrocellulose membranes for Western immunoblot analyses using a monospecific MG anti serum (Biovac, Beaucouz´e, France) diluted at 1/100. The secondary antibody used was anti-chicken IgY, HRP conjugate (Sigma-Aldrich, Milano, Italy) diluted at 1/1,000 (Bencina et al., 1994). After the immunoblot, the Comassie gel and the blotted membrane were com pared in order to detect immunoreactive proteins.

Protein Identification

The spots selected by immunochemical analyses were subjected to MALDI–TOF/TOF mass spectrom etry for identification using an Ultraflex III MALDI TOF/TOF instrument (Bruker Daltonics, Bremen). The spots were cut out of the 2-DE, destained overnight with a 4.2 g/L solution of ammonium bicarbonate and 400 mL/L ethanol, and then washed 3 times for 10 min with acetonitrile and dried in a Speedvac. Proteins were in gel digested with trypsin (Promega, Madison, WI), and spectra were acquired as previously described (Zava et al., 2009). Mascot and MS-Fit software packages were used to search against the NCBInr.2011.10.10 database using the peptide-mass fingerprinting (PMF) method (Pappin et al., 1993). The following parameters were set for the searches: S-carbamidomethyl derivate on cys teine as fixed modification, oxidation on methionine as variable modification, and 2 missed cleavage sites for trypsin digestion. The peptide mass tolerance was 20 ppm.

For in silico analysis, the Basic Local Alignment Search Tool (BLAST) program (Altschul et al., 1990) was used for nucleotide and protein homology search, as far as for the detection of tryptophan codons.

Site-directed Polymerase Chain Reaction-Mediated Mutagenesis

Mycoplasma gallisepticum American Type Culture Collection (ATCC) Strain 15302 was used for genomic DNA extraction.

A single tryptophan codon UGA was detected in the whole E2 gene and point-mutated to convert into UGG, using two polymerase chain reaction (PCR) runs as described (Villarroel and Regal ado, 1997; Rosati et al., 2000). Briefly, in the first run, the following primer sets (mutated positions are boxed) were used to generate 2 overlapping fragment [offset by 30 base pair (bp)]: MGfow 5′ TTGGATCCTTTGAATATAAATTTACAGACGTTG and MGmut– rev 5′ -TTATCAGCACCATCGAT CA TCTGTGGTCAGCTGC for the amplification of the first fragment and MGmut– fow 5′ -TTGCAGC TGACCACAGAT GATCGATGGTGCTGAT and MGrev 5′-TTGAATTCAATTAGTAAACCGTTAAGA TTTTC for the second fragment. The MGfow and MGrev primers contained restriction sites for BamHI and EcoRI, respectively (underlined in the primer sequences), to facilitate the following cloning step.

Forward MGfow primer was designed excluding the protein leader peptide sequence. The first PCR reaction (50 µL volume) contained 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM deoxynucleotide triphosphates (dNTPs), 10 pmol each primers, 50 ng of template, 1 U Hotstar Taq DNA polymerase (Qiagen, Venlo, Netherlands). PCR was performed in 7min at 95◦C followed by 30 cycles of 60 s at 95◦C, 60 s at 50◦C, and 60 s at 72◦C, and a final extension of 7min at 72◦C. Amplified products were resolved and visualized after agarose gel electrophoresis and ethidium bromide staining, gel purified, and combined in equimolar concentrations to form the template for the second PCR run, using the most external primers. During the annealing step, overlapping fragments joined each other accomplishing the full length synthesis of the gene encoding the mature protein. The second reaction mixture (50µL volume) contained the same buffer as above, 200µM dNTPs, 50pmol each primer, and 1 U Taq polymerase. Amplification was performed as described above in 40 cycles.

Cloning and Sequencing of Mutated Genes

Amplified product of the expected length was di gested with appropriate restriction enzymes (Thermo Scientific, Waltham, MA), purified, and cloned into pSER-6HIS expression vector, which allows expression of the recombinant protein fused to a 6× His tag at the N-terminus. The recombinant plasmid was used to transform Escherichia coli BL21 (i.e., DE3) competent cells, plated on Luria–Bertani (LB) agar with ampi cillin (50µg/mL) and screened by colony PCR, using primers specific for the pSER vector (Robino et al., 2005). Positive colonies were grown in 2mL LB with ampicillin (50µg/mL). Plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen, Venlo, Nether lands) and sequenced in order to confirm the induced point mutation (BMR Genomics, Padova, Italy).

Expression and Purification of Recombinant Protein

To express the recombinant protein, early log phase culture [optical density (OD)600 = 0.6] of a positive clone was induced for 2 h with 0.1mM isopropyl b thiogalactopyranoside (IPTG) under agitation. Bacte rial cells were recovered by centrifugation, washed 1× with PBS, and lysed by conventional physico-chemical methods. Soluble fractions were separated by centrifu gation and sequentially extracted in a buffer contain ing 20mM sodium phosphate pH 7.4, 0.5M NaCl, 10mM imidazole, and increscent urea concentrations (urea 0 M : S0 fraction; urea 1M: S1 fraction; urea 4M: S2 fraction; urea 8M: S3 fraction).

Fraction analysis, carried out by SDS–PAGE, re vealed that recombinant protein was located in the S3 fraction. Purification was conducted by immobilized metal chelate affinity chromatography using an high performance liquid chromatography (HPLC) system. (GE Healthcare, Little Chalfont, UK) under denatur ing condition (Smith and Johnson, 1988). Purity of each fraction of eluted protein was analyzed by SDS–PAGE and protein concentration estimated by a DC protein assay (BioRad, Hercules, CA).

Western Blot Analysis

To confirm the presence and the molecular mass of the recombinant proteins, Western blotting was car ried out using an anti-6× His monoclonal antibody as probe. Purified recombinant proteins were sepa rated by 10% SDS–PAGE, transferred to a nitrocellu lose membrane, and subsequently blocked with 5% non fat powdered milk in PBS for 2h. After washing with PBS-Tween 0.05%, the membrane was incubated with anti-6× His monoclonal antibody (GE Healthcare, Lit tle Chalfont, UK) for 1 h at room temperature. After a second washing step, the membrane was incubated with horseradish peroxidase (HRP)-conjugate anti-mouse IgG (KPL Laboratories, Gaithersburg, UMDSA) for 1 h at room temperature. Immunoreactive protein bands were stained with 10 mg 3,3′-diaminobenzidine tetrahy drochloride (DAB, Sigma-Aldrich, Milano, Italy).

To evaluate the expression, antigenic stability, and specificity of the corresponding native protein, a spe cific rabbit antiserum was elicited using the purified recombinant antigen E2 as an immunogen (Khiari and Mardassi, 2012). The rabbit serum was probed against whole lysate of 7 different field MG strains as well as different avian mycoplasmas [2 Mycoplasma syn oviae (MS) and 1 Mycoplasma iowae (MI) samples]. All samples are field isolates that were identified and characterized by denaturing gradient gel electrophore sis (DGGE) PCR in our laboratory.

Serum Samples and ELISA Procedure

For the evaluation of the developed ELISA protocol 12 serum samples were collected from naturally MG infected chickens flocks (confirmed by PCR and bacte rial isolation) and 32 from specific-pathogen-free (SPF) subjects. ELISA was carried out as previously described with minor modifications (Rosati et al., 2000), using a commercially available monospecific MG antiserum (Biovac, Beaucouz´e, France) as positive control and a SPF serum as negative control.

Briefly, microplate was coated with 100 ng/well of re combinant antigen. Serum samples, diluted 1/10 were incubated, in duplicate, for 1h at room temperature. After 3 washing steps, 100 of anti-chicken IgY (Sigma Aldrich, Milano, Italy) was added to each well and plate incubated as above. Following the final washing steps, reactions were developed with 2,20-azino-bis-[3 ethylbenzthiazoline-6-sulfonic acid (ABTS, Chemicon, Temecuta, CA)] and read at 405 nm. Absorbance was

expressed as percentage of reactivity of the positive con trol serum. Sera with reactivity > 40% of the positive control serum were classified as positive. Differences be tween absorbance in negative and positive sera were tested by nonparametric statistics (Wilcoxon 2-sample test) using R software (R Core Team, 2013).

RESULTS

Two-Dimensional Gel Electrophoresis and Mass Spectrometry Analysis and Protein Identification

Following fractionation of total cell lysate and 2-DE, a companion gel was electro blotted on membrane and immunostained with reference MG positive sera. Two major spots were recognized (Figure 1) and analyzed by MALDI–TOF/TOF. Peptide mass fingerprinting re sulted in the identification of pMGA and E2 protein (a total of 3 peptides recognized in a total of 27 with a coverage of 11%). Since pMGA is already a well-known protein, with a large potential for generation of anti genic variants (Bencina, 2002), we decided to further characterize only the E2 protein.

Recombinant E2 Expression

The complete gene of E2, lacking the most 5′ ter minal region corresponding to the leader peptide, was then retrieved from the database Genbank amplified in 2 different PCR runs to accomplish site-directed muta genesis to convert a single TGA codon into TGG and allow expression of full product in EC. As expected, af ter the second PCR run a single band of 1.3 kb was detected. The amplicon was cloned into pSER-6HIS plasmid and sequence analysis

conducted with vector primers confirmed that mutagenesis was truly accom plished and the E2 amplicon was cloned in the correct frame and orientation. When positive clone was grown and induced, a new protein band was clearly detected in SDS–PAGE with an apparent molecular weight of 49 kDa, corresponding to E2 protein. The recombinant E2 fusion protein was found to be soluble in 8M urea and purified under denatured condition.

Western Blot and ELISA Analyses

The purified recombinant protein was immunos tained in Western Blot using an anti-6× His MAb which probes the C′ terminal tag of fusion protein. The purified recombinant protein was used as immunogen to elicit a specific immunoresponse in rabbit and the monospecific serum was used in a second Western Blot experiments using total cell lysates of different avian mycoplasmas. The antiserum was able to recognize the native protein with the expected molecular weight in all MG samples tested; meanwhile no reaction was ob served in the MS and MI control samples (Figure 2).

Recombinant E2 was tested in ELISA at the opti mal conditions described above. A panel including 12 positive and 32 negative samples was tested. Results showed a significant difference between OD recorded in the 2 groups. In more details, all positive sera tested positive in ELISA; among negative samples, 3 sera were erroneously classified as positive, showing low OD val ues. Differences between OD values of sera belonging to positive and negative sample sets were statistically significant as expected (Wilcoxon rank sum test $P < 0.001$). A boxplot of ELISA OD values obtained by r E2 ELISA is shown in Figure 3.

DISCUSSION

The identification and characterization of novel immunogenic proteins are important for the development of both improved diagnostic assays and subunit vac cines. In order to reach new and useful results in this research field, the 2-DE is a technique that can be routinely applied for parallel quantitative expression pro filing of large sets of complex protein mixtures. This technique is able to detect and characterize immunodominant proteins, and it was used to improve the knowledge about MG proteins as potential candidates for the diagnosis and control of infections (Go¨rg et al., 2004).

The pMGA gene family is a large group of surface proteins mediating the phase variation of the bacterial surface architecture and they are believed to be involved in evasion of the host immune system (Glew et al., 2000; Bearson et al., 2003; Szczepanek et al., 2010). Metabolic pathways have also been shown to contribute to MG virulence and host-interaction and, in our study, a com ponent of the pyruvate dehydrogenase was identified by 2-DE analysis, the dihydrolipoamide acetyltransferase (i.e., E2). This protein was first described by Jan and colleagues in 1996 and fully sequenced by Szczepanek and colleagues in 2010. E2 was proven to be highly im munogenic and antibodies directed against it induced growth inhibition of cells and activation of the comple ment system. Moreover, topological experiments using in situ treatment with proteases and growth inhibition in the presence of anti-E2 serum provided evidence of the surface exposition of the polypeptide (Jan et al., 1996), but to our knowledge no serological and diag nostic potential of this protein was not yet evaluated.

In this study, with the aim to fully characterize the E2, we directly mutagenized its gene and successfully produced recombinant protein in a prokaryotic sys tem. The results obtained after the Western Blot using the E2 rabbit antiserum with different strains of my coplasmas showed that E2 protein is stably expressed in all MG tested strains isolated in different parts of Italy, while no cross reaction was found in any MS and MI strains tested. This aspect is particularly impor tant given that MG is equally pathogenic for poultry compared to MS and MI, causing important economic losses. Under the diagnostic point of view it is funda mental to overcome the possibility of cross reactions in serological tests, especially in field samples. These results also agree with the ones found by Jan and coau thors in 2001. Indeed the anti-

E2 serum revealed a polypeptide with an apparent molecular mass close to 52 kDa in all of the MG strains assayed, demonstrating the antigenic stability of the protein and reinforcing the idea that it might be used to develop serologic diagnostic tests based on the detection of anti-E2 antibodies in infected hosts.

In the ELISA test, r-E2 was capable of detecting all the positive serum tested, even though there were some showing low OD values, slightly above the cutoff. The presence of 3 false-positive results among negative samples revealed a nonabsolute specificity of the test. It is known that many parameters are involved in stimulating the avian immune system and the obtained results can be explained by the variation of the immune response of the host, according to individual response to infection and heterogeneity of the antigen presentation by the MG (Levisohn et al., 1995; Grodio et al., 2012). Moreover, Western Blot supports the specificity of the

E2 against sera positive for other than MG mycoplasma strains. Given this, our results seem to suggest that the false positive outcomes could be justified by a non ideal ELISA test optimization. Further investigations about serum dilution and conjugate concentration will be per formed in order to obtain higher signal-to-noise ratio and, consequently, better diagnostic performances.

In conclusion, the protein identified by the 2-DE analysis was successfully mutagenized demonstrating that the used method is fast and highly efficient. Over all, E2 can represent a recombinant antigen with poten tial diagnostic applications, being discriminative, cost effective, and less time-consuming. Moreover, E2 can improve diagnostic tools together with the well-known pMGA and pvpA antigens, increasing the spectrum of MG antibodies detected in serological procedures and representing an important update in the diagnostic of mycoplasma infection in poultry.

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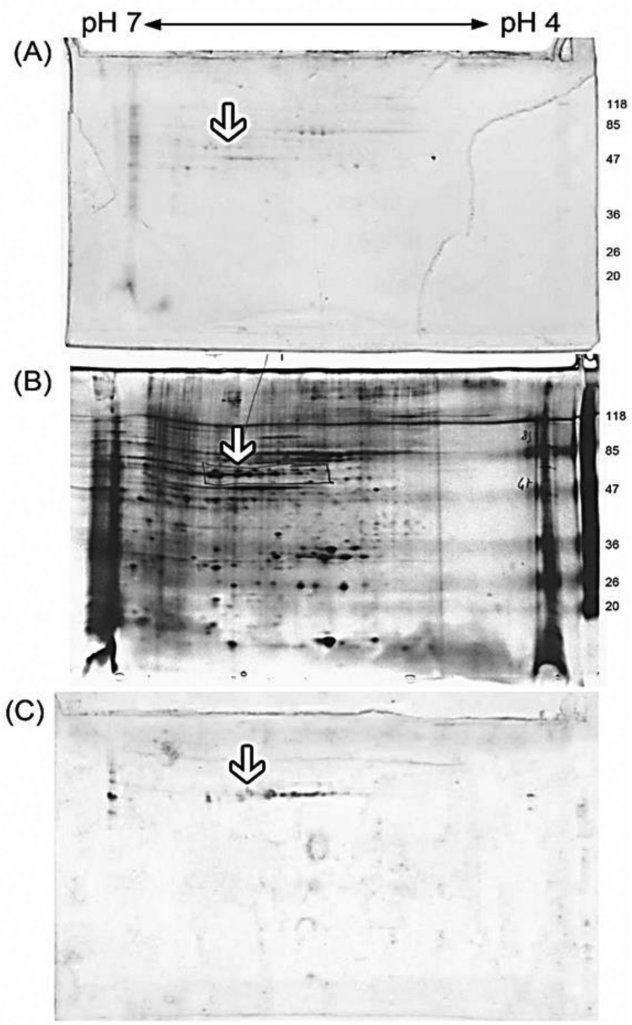

Figure 1. 2D gel electrophoresis and Western blot of *Mycoplasma gallisepticum* total proteins. MG proteins were separated on pH 4 to 7 linear strips and 10% acrylamide gels followed by (A) Coomassie staining, (B) silver staining (B), and (C) immunoblotting using monospecific serum. Proteins in the molecular mass standard are indicated to the right of the gels. Spot position of the E2 protein is indicated by white arrow.

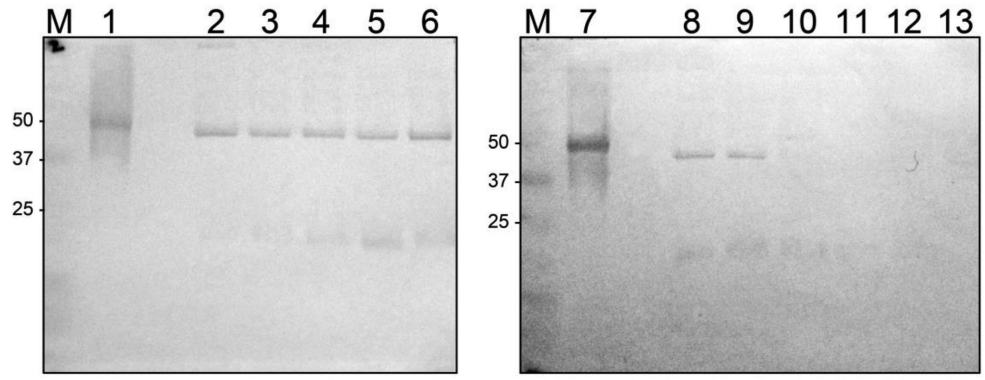

Figure 2. Western Blot using the E2 rabbit antiserum. Lanes M: molecular weight marker; Lanes 1 and 7: r-E2 (positive control); Lanes 2 to 6, 8, and 9: whole lysate of different field *Mycoplasma gallisepticum* strains; Lanes 10 and 11: whole lysate of different *Mycoplasma synoviae* strains; Lane 12: whole lysate of *Mycoplasma iowae*; Lane 13: negative control.

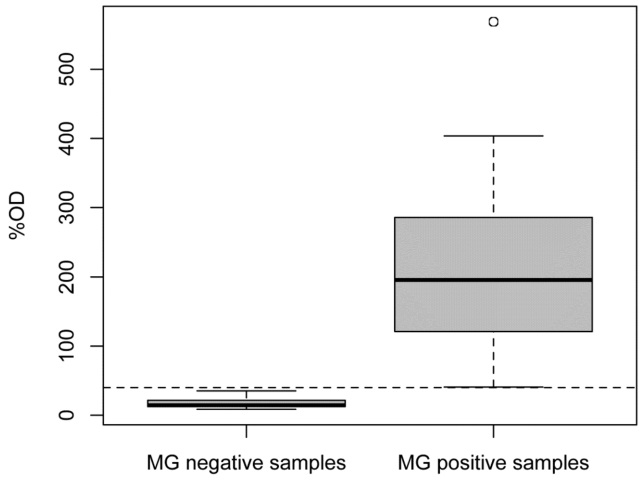

Figure 3. Distributions of D-values in ELISA test. OD values are reported as percentage of reactivity of the positive control serum.