Detection of antigenic variation among strains of poultry mycoplasma by immunoblot analysis

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Abstract

Immunoblotting analysis revealed immunodominant properties and the most intensive cross-reactivity of 39-44 kDa, 64-70 kDa and 77-80 kDa proteins of mycoplasma strains and isolates tested. Non-pathogenic M. gallinarum species isolates did not express antigenic heterogeneity. Protein polymorphism of pathogenic M. synoviae species isolates were determined by both the differences between intensity of expression of 77-88 kDa protein and the differences in size of 38-46 kDa proteins. Soluble antigen fraction and polyvalent sera testing by immunoblotting analysis enables to determine morphological or antigenic heterogeneity of strains but fails to reveal species-specific features.

Key words: *poultry mycoplasma, immunobot analysis, polyclonal antisera.*

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Introduction

Despite the advances in the diagnosis and treatment of infectious diseases, pathogenic microorganisms remain the most important threat to poultry industry. Pathogenic mycoplasmas cause diseases and loss of production especially in intensively reared poultry farms, particularly in those under environmental stress. The most important poultry pathogens are *Mycoplasma* (*M.*) *gallisepticum*, *M. synoviae* and *M. iowae* all of which continue to cause significant economic losses [1]. These pathogens may cause chronic and acute respiratory diseases, synovitis, lung and air bag inflammation, arthritis and conjunctivitis. Due to mycoplasma effect the embryonic mortality is increased, the hatchability is impaired; the production loses its market value [2, 3].

The mycoplasmas are routinely detected by serological methods, based on the specific interaction of antigen and antibody: the rapid serum agglutination (RSA) test, the hemagglutination inhibition (HI) test, enzyme-linked immunosorbent assay (ELISA). However, long ago have noticed the antigenic relationship and cross-reactions of *M. synoviae* and *M. gallisepticum* giving false positive results [4, 5].

While the most diagnostic and research work focuses on the diagnosis and testing of pathogenic mycoplasmas, more than 20 other species of genus *Mycoplasma* have been isolated, *Acholeplasma* and *Ureaplasma* among them [6, 7], also new species have been defined [8-11]. Under specific conditions their pathogenicity may be expressed [12]. In spite of their small genome size, mycoplasmas have a surprisingly great capacity of antigenic variation. The capacity of mycoplasmas to change their surface exposed antigenic determinants is thought to be crucial for their survival in immunocompetent hosts [13]. A great variety of pathogenic and nonpathogenic strains within the species, their antigenic heterogeneity and variability and the presence of mixed infections of different types make it quite difficult to determine mycoplasmas [14-16]. There is but scarce evidence in literature on the antigenic properties and possible cross-reactions of non-pathogenic poultry mycoplasmas with *M. synoviae* and *M. gallisepticum*, which may influence test results.

For vaccination live avirulent strains are used, therefore to evaluate the efficiency of the vaccination we should use the methods for identification of wild and vaccine strains of the same species [17-19]. The methods for identification of separate strains are necessary in epidemiological studies as well. In the recent years the genetic methods are applied for this purpose. The knowledge of the gene sequence changes is of practical value in studying the incidence of *M. gallisepticum* and *M. synoviae* [20-22].

Constant and intensive investigations of poultry mycoplasma will help to control the diseases caused by these pathogens. The high incidence of *M. gallisepticum*

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and *M. synoviae* among the world intensive poultry breeds shows the necessity of further studies.

The aim of the study was to determine by the method of immunoblotting the immunodominant and cross-reactive proteins of mycoplasma strains and field isolates.

Materials and methods

Mycoplasma cultures

In this study we used mycoplasma reference strains *M. gallisepticum* S6, *M. gallinaceum* D, *M. gallinarum* FR 7189 and *M. synoviae* WVU 1853 kindly provided by Prof. J. Šiugzdaitė (Lithuanian Veterinary Academy). *M. gallisepticum* 6/85 was obtained as a commercial product (vaccine strain, Intervet International B.V., The Netherlands). *M. gallinarum* field isolates VP, GP, JV, JG, *M. synoviae* VPbr and *Acholeplasma* genus GP 4-9 were isolated and purified by us in this study.

Mycoplasmic cultures were cultivated at 37°C under anaerobic condition in culture media containing 2% broth base (BBL™ Mycoplasma broth base (Frey), BD, USA), 15% heat inactivated swine serum, 2.5% yeast extract (BD, USA), 1% glucose, 0.01% cysteine hydrochloride (Acros Organics, Belgium), 0.01% reduced form β-NADH (Acros Organics, Belgium), 50 IU/ml penicillin G, 0.01% thallium acetate (Sigma, USA), 0.002% phenol red, pH 7.8. Solid medium was prepared without phenol red, but adding 1% of agar (BD, USA). Field isolates were obtained and purified from hen trachea mucous samples according to Clyde W.A.Jr. and McCormack W.M. [23].

The preparation of mycoplasmic antigens

Mycoplasma cultures were cultivated in 100-500 of liquid culture medium. Cells were collected by centrifugation (6000 × g for 30 min) at pH 6.8. The pellet was washed three times resuspending in 10 ml PBS (80 mM Na_2HPO_4, 20 mM Na $H_2PO_4 \cdot 2H_2O$, 100 mM NaCl, pH 7.2) and centrifuged at 6000 × g for 20 min. The part of cell culture that was used for immunization was diluted in PBS up to 200 µg/ml, divided into 1 ml volumes and stored at –20°C.

For sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting the antigenic preparations from the soluble fraction of mycoplasma culture were collected by lysing for 1 h at room temperature in PBS containing 25 mM Tris-HCl, 0.25% sodium deoxycholate, 1% Triton X-100, 0.05% Tween 20, 1.5 M NaCl, 0.1 M phenylmethylsulsonyl fluorid, pH 8.1. Nonsoluble components were sedimented by centrifugation at 12 000 × g 10 min. The supernatant obtained was transferred into a new tube. Protein concentration was determined by an improved Lowry assay (Bio-Rad DC Protein Assay kit, Bio-Rad Laboratories, USA). The measurements were performed using biophotometer (Eppendorf BioPhotometer, Germany) at 750 nm wavelengths.

Protein polyacrylamide gel electrophoresis

SDS-PAGE was performed in a 10% resolving gel containing a 4% stacking gel according to Laemli U.K. [24] in a Miniprotein II mini-gel system (Sigma, USA). The samples were mixed in equal volumes of the electrophoresis sample buffer (0.125 M Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 20% glycerol, 4% SDS and 0.02% bromphenol blue), heated for 5 min at 100°C. For the visual evaluation of SDS-PAGE results 20 µg of proteins were loaded onto the concentrated gel wells. For the immunoblotting analysis 10 µg of protein were loaded per well. To estimate molecular masses of peak fractions we used 5 µl of known commercial molecular size markers. Electrophoresis was performed using constant current of 6 mA for 30 min and 8 mA until the tracking dye had migrated to the bottom of the resolving gel. Polyacrylamide gels were stained by 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid aquatic solution. Gels were analyzed by BioDocAnalyzer (Biometra, Germany).

Production of polyvalent antisera and immunoglobulins

The work was performed with the consent of the Ethics Commission on the Use of Laboratory Animals and the Lithuanian State Veterinary and Food Service to use rabbits in our experiments (the permission No. 0086).

To prepare the control sera, blood samples were collected from 7 rabbits before immunization. Later rabbits were immunized as described by Senterfit L.B. [25]. 200 µg of antigens in 1 ml PBS were emulsified with Freund's complete adjuvant (1 : 1). Rabbits were immunized by intramuscular (0.4 ml of suspension) and intradermal (0.1 ml to four sites each) inoculations. At 3-week interval the immunization was repeated. Following the second immunization at 21, 24 and 27 days rabbits were inoculated in the ear vein by 200 µg of antigen. The blood was collected after 1 week following the last immunization. For immunoblotting immunoglobulin (Ig) fraction from antisera was sedimented by adding equal volume of saturated sulphate of ammonia solution, the 6 hours incubation at 4°C and by centrifugation (6000 × g for 10 min). Supernatant was removed and Ig fraction resuspended in 1 ml PBS and equal volume of saturated sulphate of ammonia solution. Protein concentration was determined using Bio-Rad DC Protein Assay Kit.

Immunoblot analysis

Polypeptides from SDS-PAGE were transferred to 0.45 µm Hybond-P PVDF membranes (Amersham Biosciences) as described by Kyhse-Anderson L. [26] using MilliBlot™-Graphite Electrobloter apparatus (Millipore, USA) at 200

mA 30 min. Membranes with the transferred polypeptides were blocked for 1 h with PBS containing 3% BSA and 0.1% Tween 20 and then exposed for 1 h to the specific rabbit Ig diluted in washing buffer (PBS, 0.1% Tween 20), for 1 h to peroxidase-conjugated anti-rabit whole antibody from donkey (Amersham Biosciences) diluted in washing buffer. Blots were given six 5-min rinses with washing buffer between each step. Blots were developed with ECL Western blotting detection reagents and analysis system following manufacturer's (Amersham Biosciences) instructions.

Results

Immunodominant and croossreacting with another strain specific Ig fraction mycoplasma proteins were determined by the technique of immunoblotting. The experimental conditions were optimized in every test, choosing the optimal concentrations of sedimented immunoglobulins and conjugate to have a maximal number of interacting protein bands.

M. gallisepticum 6/85 strain-specific Ig were cross reactive with the all studied strains and isolates of 64-70 and 38-44 kDa protein fractions (Fig. 1). The highest intensity of the Ig reactivity was found with *M. gallisepticum* 6/85 strain familiar 123, 104, 84, 66, 52, 48, 35, 28 kDa molecular mass or less than 19 kDa molecular mass proteins. More active interaction of 39-44 and 31-35 kDa antigens was noticed (Fig. 1, lane 1). A rather intensive interaction of *M. gallisepticum* S6 strain 75, 64, 59, 52, 48, 41-44, 30-33 kDa and (with) *M. gallisepticum* 6/85 specific Ig (Fig. 1, lane 2) was found.

The most significant interaction of specific Ig was registered with *M. gallisepticum* S6 strain 75, 56-59, 41-52; slightly lower – with 129, 114, 101, 84, 38, 33, 25 and 20 kDa protein fractions (Fig. 2, lane 2). This strain specific Ig interacted with the all mycoplasma strains and isolates of 40-43 kDa fraction (Fig. 2).

The highest immunogeneity was found in 103-155, 80, 66, 60, 46 and 37-41 kDa molecular weight protein fractions of *M. gallinaceum* D (Fig. 3, lane 3). This strain specific polyvalent Ig cross reacted intensively with all tested strains and isolates of 105-155, 77-80, 39-40 and 66 kDa fraction of VPbr-39 isolate (Fig. 3).

The most expressed binding of *M. gallinarum* FR 7189 specific Ig was found with 108-104, 77-86, 66, 58, 55, 49, 40-43 and 38 kDa proteins (Fig. 4, lane 4). Cross reactivity of these Ig was found with 112, 105, 84 and 52 kDa fractions of *M. gallisepticum* 6/85 strain (Fig. 4, lane 1), 75-80, 56 and 46 kDa fractions of *M. gallisepticum* S6 strain (Fig. 4, lane 2) and 112, 105, 98, 80, 66, 59, 54, and 41 kDa protein fractions of *M. gallinaceum* (Fig. 4, lane 3). *M. gallinarum* FR 7189 strain specific Ig interact with the high molecular weight GP 4-9, 80-84 kDa fractions of VPbr isolates (Fig. 4, lane 5) and 98, 86, 77 kDa fractions of the same isolate (Fig. 4, lane 6).

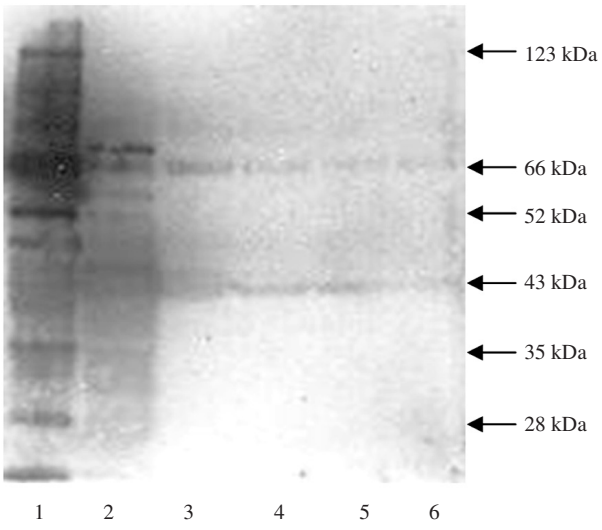

Fig. 1. Immunoblot analysis of poultry mycoplasma proteins using Ig fraction from rabbit serum against *M. gallisepticum* 6/85

1 – M. gallisepticum 6/85, 2 – M. gallisepticum S6, 3 – M. gallinaceum D, 4 – M. gallinarum FR 7189, 5 – GP 4-9, 6 – VPbr 21-39 soluble protein fraction. Concentration of rabbit Ig – 1 µg/ml, dilution conjugated Ig – 1 : 40 000.

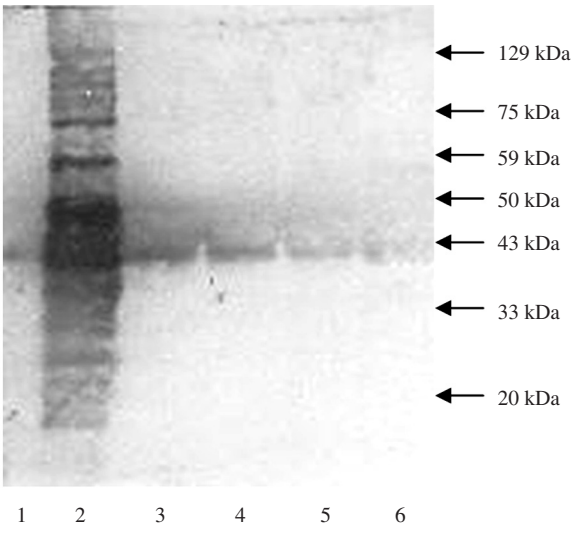

Fig. 2. Immunoblot analysis of poultry mycoplasma proteins using Ig fraction from rabbit serum against *M. gallisepticum* S6

1 – M. gallisepticum 6/85, 2 – M. gallisepticum S6, 3 – M. gallinaceum D, 4 –M. gallinarum FR 7189, 5 – GP 4-9, 6 – VPbr 21-39 soluble protein fraction. Concentration of rabbit Ig – 1 µg/ml, dilution conjugated Ig – 1 : 40 000.

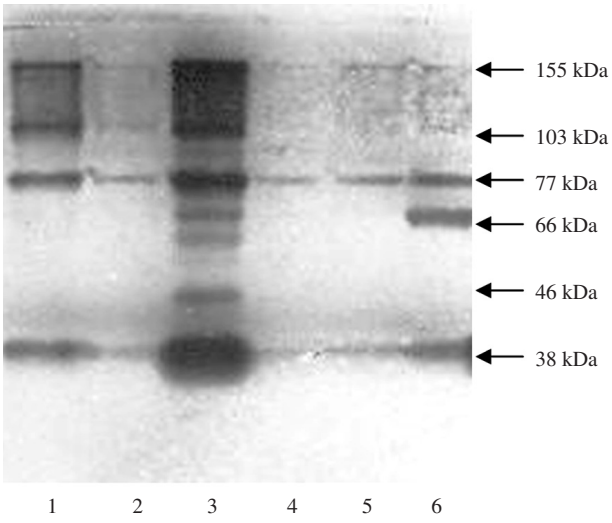

Fig. 3. Immunoblot analysis of poultry mycoplasma proteins using Ig fraction from rabbit serum against *M. gallinaceum* D
1 – M. gallisepticum 6/85, 2 – M. gallisepticum S6, 3 – M. gallinaceum D, 4 – M. gallinarum FR 7189, 5 – GP 4-9, 6 – VPbr 21-39 soluble protein fraction. Concentration of rabbit Ig – 2 µg/ml, dilution conjugated Ig – 1 : 100 000.

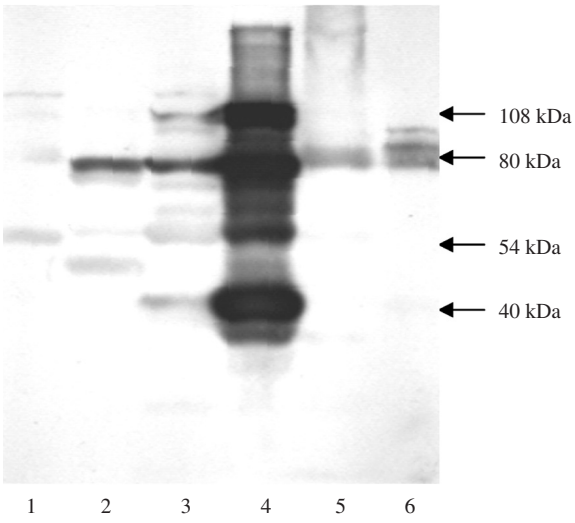

Fig. 4. Cross-reactive proteins among poultry mycoplasmas detected by Ig fraction from rabbit serum against *M. gallinarum* FR 7189 in immunoblot analysis
1 – M. gallisepticum 6/85, 2 – M. gallisepticum S6, 3 – M. gallinaceum D, 4 – M. gallinarum FR 7189, 5 – GP 4-9, 6 – VPbr 21-39 soluble protein fraction. Concentration of rabbit Ig – 3 µg/ml, dilution conjugated Ig – 1 : 100 000.

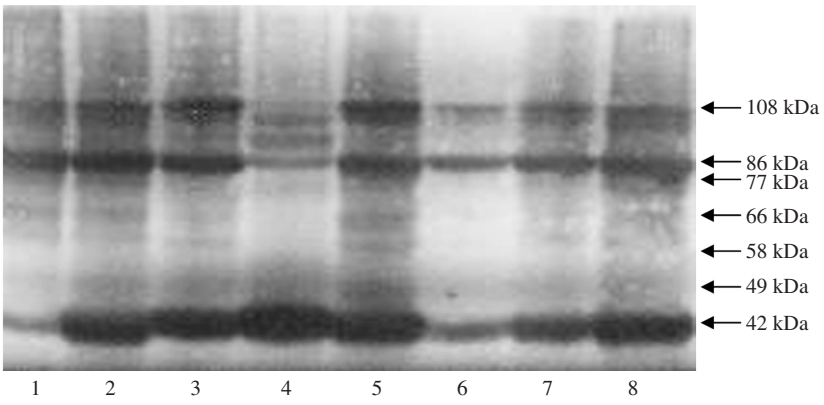

Fig. 5. Immunoblot analysis of field isolates using Ig fraction from rabbit serum against *M. gallinarum* FR 7189
1 – VP 22-2, 2 – VP 30-5, 3 – GP 22-1, 4 – GP 23-3, 5 – GP 23-4, 6 – JV 9-1, 7 – JV 9-5, 8 – JG 7-1 soluble protein fraction. Concentration of rabbit Ig – 2 µg/ml, dilution conjugated Ig – 1 : 100 000.

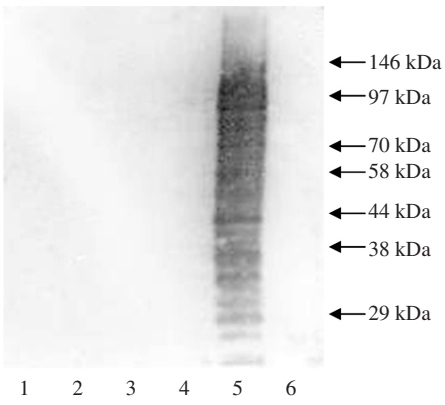

Fig. 6. Immunoblot analysis of poultry mycoplasma proteins using Ig fraction from rabbit serum against GP 4-9
1 – M. gallisepticum 6/85, 2 – M. gallisepticum S6, 3 – M. gallinaceum D, 4 – M. gallinarum FR 7189, 5 – GP 4-9, 6 – VPbr 21-39 soluble protein fraction.

The results on VP, GP (except GP 4-9), JV and JG protein isolates obtained in our test by immunoblotting using *M. gallinarum* FR 7189 strain specific serum Ig (Fig. 5) coincided with the results obtained using *M. gallinarum* FR 7189 specific serum Ig (Fig. 5, lane 4). The highest reactivity was found between the proteins of similar molecular weight, i.e. 104-108, 77-86, 66, 58, 47-49, 40-43 kDa.

Acholeplasma genus GP 4-9 isolate specific Ig interacted only with its proteins. The Ig concentration of 1 µg/ml used in immunoblotting (conjugate diluted at 1 : 40 000) gives the background of Ig lines with 146, 109, 97, 87, 84, 70, 58, 50, 44, 42, 38, 35, 34, 31, 29 and 26 kDa protein fractions and lower than 19.5 kDA molecular weight proteins (Fig. 6, lane 5).

Immunoblot analysis using *M. synoviae* species VPbr 21-39 isolate specific polyvalent Ig, reveals its interaction with 134, 105, 77-86, 65, 58, 46-54, 40-46, 34-37 and 24 kDa protein fractions (Fig. 7 A, lane 6) of this isolate. These Ig cross-reacted with the other used in our study mycoplasma proteins of 78-86 and 41-43 kDa (except *M. gallisepticum* 6/85) (Fig. 7 A, lanes 1-5), cross react with *M. gallinaceum* D strain, molecular mass at 66 kDa (Fig 7, lane 3) and *M. gallinarum* FR 7189 strain proteins at 77 and 47-55 kDa (Fig. 7, lane 4).

When the Ig concentration was decreased to 5 µg/ml, the reactivity was found only with VPbr 21-39 isolate protein fractions of 86, 66, 46, 40-43 and 24 kDa (Fig 7 B). The mostly expressed is 43 kDa protein fraction. Analogical experimental conditions using *M. synoviae* WVU 1853 strain specific Ig, revealed their interaction only with 86 and 43 kDa molecular mass protein fractions of VPbr 21-39 isolate (Fig. 7C).

In immunoblot assay studying the cross-reactivity of separated by SDS-PAGE proteins of VPbr isolates with VPbr 21-39 specific Ig (at the concentration of 5 mg/ml, conjugate diluted 1 : 40 000), some differences in the reactivity of separate isolates proteins according to their molecular mass were detected: though the majority of VPbr isolates expressed the highest reactivity at 43-46 kDa, VPbr 22-49 immunoblot sample revealed the profiles at 40-43 kDa (Fig. 8A, lane 5). VPbr 16-33 isolate (Fig. 8A, lane 1) and VPbr 5-55 isolate (Fig. 8A, lane 7) showed the most reactivity at 38-40 kDa. The samples of VPbr isolate soluble proteins in SDS-PAGE at the molecular mass of 35-46 kDa were differing by the number of visible bands and their distribution (Fig. 8B). In Fig. 8 B white line marked protein fractions gave the most expressed reactivity with species-specific polyvalent Ig.

The immunoblot samples of VPbr 19-33 and VPbr 5-55 share one uncommon feature to other samples, namely, the expressed binding of 21 kDa protein fraction with Ig (Fig. 8A, lanes 1, 7).

In the immunoblot sample provided above, different intensity bands of higher molecular mass 77-98 kDa proteins are seen. We suppose that this polymorphism can be possibly due to the adaptation of field isolates to cultivation conditions *in vitro*. Subpopulations from

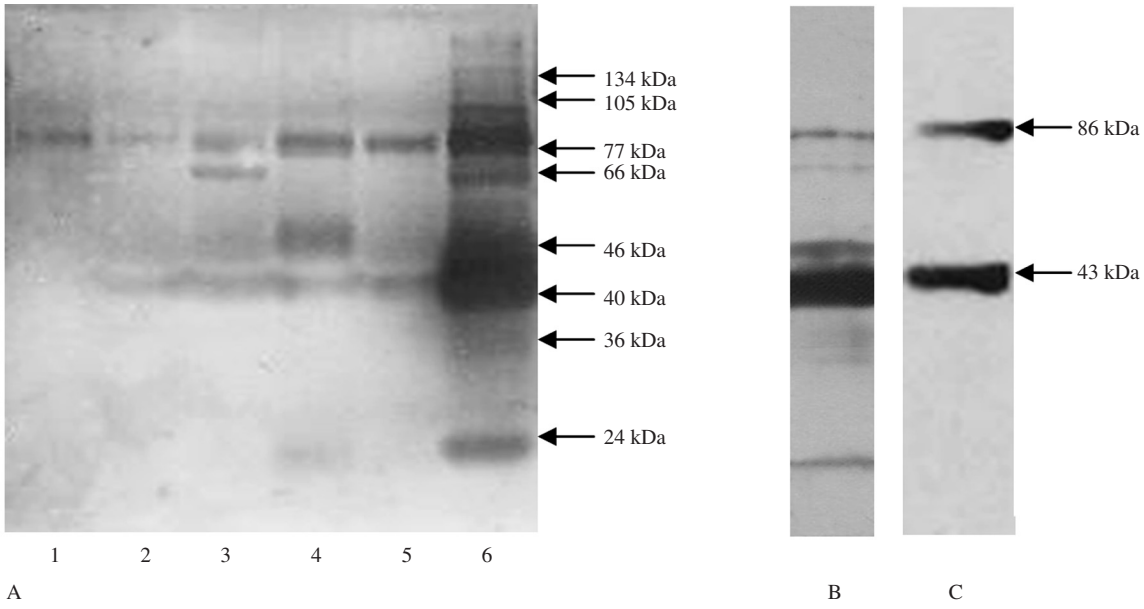

Fig. 7. Immunoblot analysis of poultry mycoplasma proteins using Ig fraction from rabbit serum against M. synoviae. A – analysis was performed using 10 µg/ml concentration rabbit Ig against VPbr 21-39 field isolate. B – VPbr 21-39 soluble protein fraction immunoblot analysis using 5 µg/ml concentration Ig fraction from homologous antisera. C – VPbr 21-39 soluble protein fraction immunoblot analysis using 5 µg/ml concentration Ig against *M. synoviae* WVU 1853

1 – M. gallisepticum 6/85, 2 – M. gallisepticum S6, 3 – M. gallinaceum D, 4 – M. gallinarum FR 7189, 5 – GP 4-9, 6 – VPbr 21-39 soluble protein fraction. Dilution conjugated Ig – 1 : 100 000

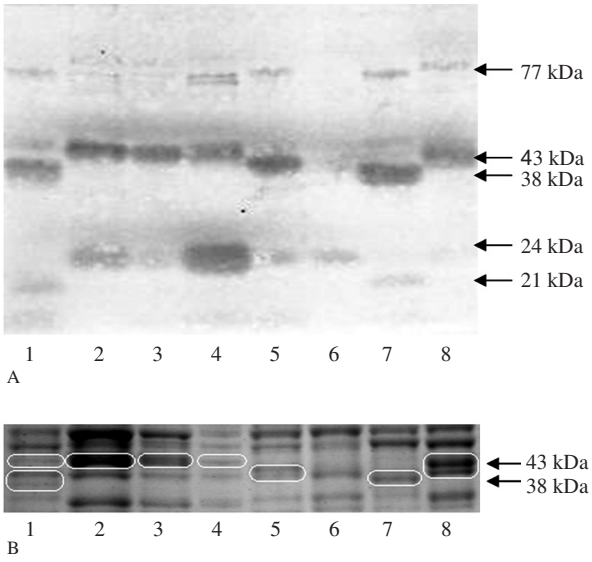

Fig. 8. Immunoblot analysis of VPbr field isolate proteins using Ig fraction from rabbit serum against VPbr 21-39 field isolate
A: 1 – VPbr 16-33, 2 – VPbr 17-36, 3 – VPbr 17-36, 4 – VPbr 26-42, 5 – VPbr 22-49, 6 – VPbr 22-49, 7 – VPbr 5-55, 8 – VPbr 22-48 soluble protein fraction. Ig concentration – 5 µg/ml
B: Fragments of soluble protein fraction profiles of field isolates (in the same course as in A) separated by SDS-PAGE

different colonies differed by the growth rate and biomass outcome. Their growth was not synchronized. *M. synoviae* expresses flexible surface immunodominant proteins, as e.g. 80 kDa hemadsorption associated protein and 45-80 kDa variable size proteins [27].

Discussion

Immunoblot method of identifying avian mycoplasma immunodominant and cross-reactive antigens derived from strains of antigenic heterogeneity of the data supporting. Studied strains and isolates characterized by different molecular weight antigens imunodominant. Revealed extensive *M. gallisepticum* S6 strain 64 and 75 kDa antigen cross-group interaction with vaccine 6/85 strain specific Ig. Immunoblot analysis shows a weak vaccine 6/85 strain of antigen interaction with the S6 strain specific serum – the reaction showed only a uniform intensity of the serum cross-interaction with all tested strains of 39-42 kDa protein fractions.

M. gallinaceum D strain-specific serum, although not actively interact with all tested mycoplasma strains and isolates 39-40, 77-80, 103 and 155 kDa antigen groups, suggesting that all tested mycoplasma antigens in these groups share a common epitope. *M. gallinaceum* D specific serum interacted only with the homologous strain 46 and 60 kDa antigen fractions of antigenic specificity, apparently limited to that specific strain or species.

Immunodominant distinguished characteristics of M. gallinarum FR 7189 strain, as these types of securities, GP, JV, JG 108 isolates, 40-43, 58 and 86 kDa antigen fractions. The data suggest that this type of immunogenic protein isolates uncharacteristic amount of variability. Matching immunoblot analysis suggests that the isolates belong to the same strain FR 7189. Intensifying the strain-specific cross-Ig interacted with *M. gallisepticum* S6, *M. gallinaceum* D strains and GP 4-9 isolate 80 kDa and VPbr 21-39 isolate 77 and 86 kDa antigens.

In immunoblot study do not find the GP 4-9 (Acholeplasma genus) isolate specific serum cross-reactivity. Not revealed this isolate is divergent characteristics immunodominant group antigen - serum Ig bound to a similar intensity of a number of less than 146 kDa molecular weight antigen fractions.

M. synoviae type VPbr 21-39 isolate 43 and 86 kDa antigen groups interact intensively with both the homologous as well as with *M. synoviae* WVU 1853 strain-specific serum Ig. With homologous serum Ig also actively responded to this isolate 24, 46 and 66 kDa antigens.
Found for all tested mycoplasma 41-43and 78-86 kDa, and *M. gallinaceum* D strain, 66 kDa and *M. gallinarum* FR 7189 strain 47-55 kDa antigen fractions of cross-reactivity with VPbr 21-39 isolate specific Ig. Immunoblot analysis revealed higher *M. gallisepticum* and *M. synoviae* antigenic relatedness of species.

By immunoblotting *M. synoviae* field isolate protein with the VPbr 21-39 isolate specific polyvalent Ig has been studied. The highest binding was found in samples with the protein fraction from 38 to 46 kDa. It confirmed using in immunoblotting *M. synoviae* infected poultry serum or vlhA gene encoded MSPA and MSPB protein specific monoclonal or polyclonal antibodies [17, 21, 28]. Conservative 5'-end of the vlhA gene (nucleotides 1-410 starting from ATG code in *M. synoviae* WVU 1853) is unique for M. synoviae species. Its detection and sequence analysis is useful for strain differentiation, whilst the number of repeated sequences encoding proline-rich repeats region of MSPB protein could be associated with pathogenic properties of the strain [21].

The comparison of separate immunoblotting results revealed the highest rate of cross-reactivity in 77-80 kDa (*M. gallinaceum* D and VPbr 21-39 specific Ig), in 64-70 kDa (*M. gallisepticum* 6/85 specific Ig) and in 39-44 kDa (*M. gallisepticum* 6/85 and S6, *M. gallinaceum* D and VPbr 21-39 specific Ig) molecular mass antigens.

Soluble antigen fraction and polyvalent sera testing by immunoblotting analysis enables to determine morphological or antigenic heterogeneity of strains but fails to reveal species-specific features.

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