

**EVALUATION OF IMMUNOLOGICAL STATUS OF HORSES
AGAINST INFLUENZA VIRUS BASED ON THE PRESENCE
OF ANTIBODIES AGAINST NS1 AND M1 PROTEINS**

WOJCIECH ROŻEK, MIROŚLAW P. POLAK AND JAN F. ŻMUDZIŃSKI

Department of Virology,
National Veterinary Research Institute,
24-100 Pulawy, Poland
e-mail: wrozek@piwet.pulawy.pl

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The purpose of the study was to evaluate the immunological status of horses against influenza virus, based on the presence of antibodies against NS1 and M1 proteins. NS1 and M1 proteins have been purified from infected MDCK cell culture and from virions multiplied in embryonated chicken eggs, respectively. Immunoblotting was done with the sera from animals of unknown immunological status. The reaction enabled the detection of antibodies against influenza virus and differentiation of horses vaccinated and supposedly recovered from the infection.

Key words: horses, influenza virus, NS1 protein, M1 protein, diagnosis.

Influenza is one of the most important equine respiratory diseases. Two subtypes of equine influenza virus A/equine-1 (H7N7) and A/equine-2 (H3N8) have been identified. The latter subtype is regarded as the only causative agent of all outbreaks of the disease since the beginning of the 80's (13, 14). Acute respiratory disease with fever, depression, nasal discharge and coughing typical for influenza is frequently accompanied by secondary bacterial infections with more severe outcomes especially in non-vaccinated animals. Intense international trade and competitions facilitate introduction of infected horses into new environments. Before mass-scale vaccinations, rapid transmission of the disease was observed in young horses and in horses kept in large herds. Due to several influenza epizootics in equine population worldwide and economic losses thereupon, the prevention by vaccination became obligatory in some countries (2, 13,).

Besides horses influenza viruses of type A infect many other animal species and humans. Ten well-characterised proteins are synthesised during the influenza A virus replication in infected cells (PB1, PB2, PA, NA, HA, NP, M1, M2, NS1, NS2) (reviewed in 6). Nine proteins are packaged into virions. Two of them, M2 and NS2, were primarily regarded as the nonstructural proteins but further experiments confirmed their presence in virions (5, 15,). M1 protein is the most abundant influenza virus protein (about 3 000 copies per virion). It builds up the layer between viral membrane and the nucleocapsid. This protein interacts with membrane glycoproteins

and ribonucleoprotein (RNP) complexes. M1 protein is present both in the cellular nucleus and in the cytoplasm of infected cells. This protein probably plays regulatory functions in the intracellular transport of viral proteins (export of RNP from the nucleus to the cytoplasm) (6, 9).

NS1 protein remains the only nonstructural protein of influenza A virus. It is abundantly synthesised in infected cells from the early stages of infection. It can be found in the nucleus, nucleolus and also in the polysomal fraction. NS1 protein binds to poly(A) sequences and inhibits the nuclear export of viral and cellular mRNAs (12). It also inhibits pre-mRNA splicing. NS1 is an important regulatory protein involved in RNA turnover in infected cells (3).

Immunoprophylaxis of equine influenza is based on inactivated vaccines. Protection against infection is conferred by antibodies against surface glycoproteins, haemagglutinin and neuraminidase, which can neutralise the virus. Current diagnosis of equine influenza is based on serological testing where anti-haemagglutinin antibodies are detected (8). Antibodies to NS1 protein are only present after viral infection, therefore vaccinated animals should be devoid of such antibodies (1). M1 protein can induce antibodies both after vaccination and after influenza virus infection. Therefore NS1 and M1 proteins can be used to differentiate vaccinated and infected horses. From practical point of view this is very useful when only single serum samples are available from convalescent animals for testing (lack of paired sera in imported animals during quarantine) (11). Also repeated vaccinations lead to prolonged high HI titres, which may not be significantly boosted after natural infection, complicating classical serological diagnosis (four-fold antibody titre increase) (1). Possibility of detecting anti-M1 and anti-NS1 antibodies in one assay would provide information about current virus circulation and vaccination status of a single horse and the herd as well. In this study we tried to identify horses recovered from infection and vaccinated on the basis of presence of NS1 and M1 antibodies.

Material and Methods

Purification of M1 protein. Equine influenza A/equi/Kentucky 81 viral strain was used. 11-day old chicken embryos were inoculated with A/equi/Kentucky 81 (A2) virus. Allantoic fluids have been collected within 72 h after inoculation and centrifuged at 15 000 g for 30 min at 4°C. Supernatant was ultracentrifuged at 160 000 g for 2 h at 4°C and the pellet was resuspended in PBS pH 7.2. Sedimented virions were sonicated in an ice bath two times for 30 s each with 9.5 mm probe at 100 W, 8µ amplitude. Virions were collected by ultracentrifugation, 160 000 g for 2 h at 4°C through the layer of 40% sucrose in PBS (w/v). Viral pellets were resuspended in PBS, sonicated and ultracentrifuged as above. The final sediment was resuspended in SDS PAGE sample buffer (60 mM Tris/HCl pH 6.8, 2% SDS, 10 % glycerol, 0.001% bromphenol blue with or without 100mM dithiothreitol for reducing (+DTT) or non-reducing (-DTT) conditions respectively) and boiled for 5 min. Proteins from purified virions were separated in SDS PAGE in 15% gels. Electrophoresis was carried out at constant current of 30 mA. Coomassie blue R250 was used for gel staining. Preparative electrophoresis of M1 protein was carried out in 2 mm thick gels in non-reducing conditions. After staining, the band of M1 protein was cut out from the gel. Pieces of gel have been homogenised with Ultraturax homogenizer at 20 000 rpm for 3 min in SDS PAGE sample buffer with 100 mM dithiothreitol. Samples have been incubated

for 10 min in a boiling water bath before loading on the gel. Prestained 35.5 kDa molecular mass marker was added to the sample. The second step of preparative electrophoresis was done in 491 Prep Cell (BioRad), with a gel of 37 mm diameter. Electrophoresis was run at constant power of 12 W. Collection of 1 ml fractions at 0.7 ml/min flow rate started when the dye reached the end of the gel and it continued until the molecular weight marker reached the end of the gel as well.

Purification of NS1 protein. MDCK cells were grown in MEM Eagle supplemented with 5% foetal bovine serum and 20 mM L-glutamine at 5% CO₂ and 37°C. One-day old monolayer was covered with allantoic fluid from chicken embryos inoculated with A/equi/Kentucky 81 diluted 1/10 in PBS. HA titre in fluid was 128. MDCK cells covered with allantoic fluid from non-infected chicken embryos were used to prepare negative controls for western blotting. After 45 min of adsorption the inoculum was replaced with MEM Eagle without serum. Infected MDCK cells have been scraped into fluid after 24 h of incubation and centrifuged at 15 000 g, at 4°C for 30 min. The sediment was resuspended in SDS sample buffer (-DTT) or (+DTT) before electrophoresis. When preparing material for chromatography, the sediment of infected cells was resuspended in lysis buffer (9 M urea, 1% NP 40, 1 mM DTT, 1 mM PMSF, 20 mM Tris/HCl pH 7.8 with addition of protease inhibitors (Complete™, Boehringer Mannheim)). Then the cells have been sonicated ten times for 20 s each in an ice bath, with 9.5 mm probe at 100 W, 8 μ amplitude. The lysate was filtered through 0.22 μm filter and loaded on the Q sepharose FF (Pharmacia) equilibrated with 9 M urea, 1% NP 40, 1 mM DTT in 20 mM Tris/HCl pH 7.8. The column was washed with 20 mM Tris/HCl pH 7.8, 1 mM DTT and bound proteins have been eluted in linear gradient from 0.0 to 1.0 M NaCl in 20 mM Tris/HCl pH 8, 1 mM DTT. All steps were carried out at 4°C. Collected fractions containing NS1 protein were pooled and subjected for two step preparative electrophoresis (-DTT/ +DTT) as described above for M1 protein.

Immunoreagents used in Western blotting. Monoclonal antibody GA2B for the detection of M1 protein was bought from Serotech (cat. No. MCA401). NS1 rabbit antiserum was kindly supplied by Prof. B. Szewczyk from Medical University of Gdansk, Poland. Guinea pig serum originated from animals immunised with inactivated commercially available vaccine Gripovac, Biowet Pulawy, Poland. Serum from horse experimentally infected with A/equi/ Kentucky 81 was kindly supplied by Prof. E. Wisniewski from the Department of Horse Diseases, Nat. Vet. Res. Inst., Bydgoszcz, Poland (4). Equine sera were taken under field conditions from 6 randomly selected horses in one of the studs. F(ab')₂ fragments of IgG conjugated with horseradish peroxidase specific for mouse, rabbit, guinea pig or horses IgG Fc fragments from Jackson ImmunoResearch (cat. No. 315-036-046, 111-036-046, 106-0036-003, 308-036-008) were used as secondary antibody in immunoblot tests.

Western blotting. After SDS PAGE in 15% gels viral proteins were electrotransferred in 20 mM Tris, 192 mM glycine pH 7.4 with 20% methanol for 60 min at 4°C and constant voltage of 150 V onto Immobilon P (Millipore). Molecular weight determination was based on the positions of prestained markers. Then membranes were incubated for 60 min in blocking buffer (5% non-fat milk in TBST pH 7.4). Another 60 min incubation was done with antibody solutions (monoclonal antibodies for M1, monovalent rabbit antibodies for NS1, serum of guinea pig or horse sera) diluted accordingly in blocking buffer (M1 and NS1 antibodies - 1 : 10 000, sera of guinea pigs and horses - 1 : 1 000). After 3 washes in TBST 10 min each, membranes were incubated for 60 min with appropriate conjugate solutions. Conjugates of horseradish peroxidase with F(ab')₂ fragments of IgG specific for Fc

fragments of IgG from rabbits, mice, guinea pigs or horses (Jackson Immunoresearch), diluted 1:20 000 in TBST were used. The membranes have been washed 5 times in TBST (10 min each wash). Chemiluminescent detection system ECL Plus and ECL Hyperfilm (Amersham) have been used for signal detection.

Other methods. Lowry method for protein determination after TCA precipitation was used (Protein assay kit - Sigma P 5656) (7).

Haemagglutination inhibition test was performed according to OIE Standard Procedure (8).

Results

Purification of M1 protein. Virions purified from amniotic fluids of chicken embryos infected with A/equi/ Kentucky 81 were electrophoresed in reducing and non-reducing conditions (Fig. 1). In reducing conditions HA2 and M1 proteins migrated at the level of about 30 kDa. In non-reducing conditions HA2 and HA1 formed complex HA0 which migrated at the level of 75 kDa. M1 protein, which is monomeric, migrated at the same level in both reducing and non-reducing conditions. Therefore, for the purification of M1 protein, two subsequent stages of preparative electrophoresis in non-reducing and reducing conditions were used. Protein band obtained after electrophoresis in 15% gel at non-reducing conditions at the level of 30 kDa was cut out. M1 protein present in this band was subjected to preparative electrophoresis (+DTT) combined with constant electroelution. In total 96 fractions 1ml each have been collected. Samples of those fractions have been checked in SDS PAGE (+DTT) in 15% gel (data not shown). Fractions containing M1 protein have been pooled.

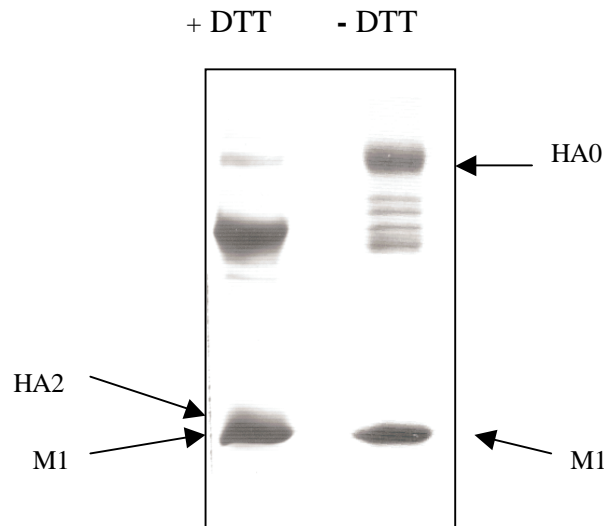


Fig. 1. SDS PAGE electrophoretic patterns comparison of purified A/equi/Kentucky 81 virions in reducing (+ DTT) and non-reducing (- DTT) conditions in 15% gel.

Purification of NS1 protein. Proteins from MDCK cell line inoculated with influenza virus A/equi/Kentucky 81 have been subjected to SDS PAGE analysis in 15% gels in reducing (+DTT) and non-reducing (-DTT) conditions. Immunodetection of M1 and NS1 proteins have been done after electrotransfer to Immobilon P membrane (Fig. 2). NS1 protein was present on the membrane at the same level as M1 protein. The presence of reducing agent had no influence on the migration of NS1 protein, which indicates that NS1 protein of A/equi/Kentucky 81 synthesised in MDCK cells is monomeric. Therefore anion-exchange chromatography for separation of M1 and NS1 proteins was applied. Western blot was used for identification of fractions containing M1 and NS1 proteins. M1 protein did not bind to the Q sepharose (it was found in the wash fraction). NS1 protein was found in a gradient from 350 to 400 mM NaCl (data not shown). Two-step preparative electrophoresis was used for NS1 purification as described in material and methods section.

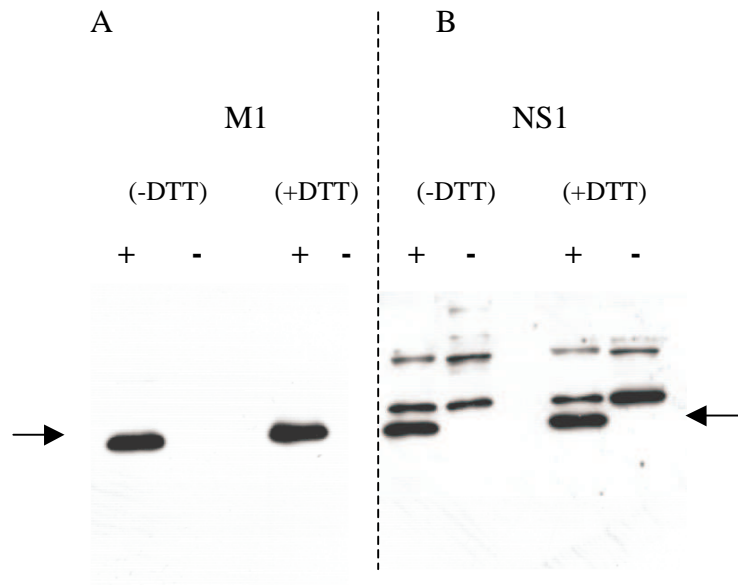


Fig. 2. Immunodetection of M1 and NS1 proteins separated in SDS PAGE in 15% gel under non-reducing (- DTT) and reducing (+ DTT) conditions.

Line + MDCK cells infected with A/equi/Kentucky 81
 Line - MDCK mock – infected cells
 (+ DTT) reducing conditions
 (- DTT) non-reducing conditions
 A immunoblot with antibodies against M1
 B immunoblot with antibodies against NS1

Detection of antibodies against NS1 and M1 proteins. Purified M1 and NS1 proteins were tested with monospecific antibodies, however, no cross-reactivity between M1 and NS1 was observed (Fig. 3). Therefore purified NS1 protein was not contaminated with M1 and *vice versa*.

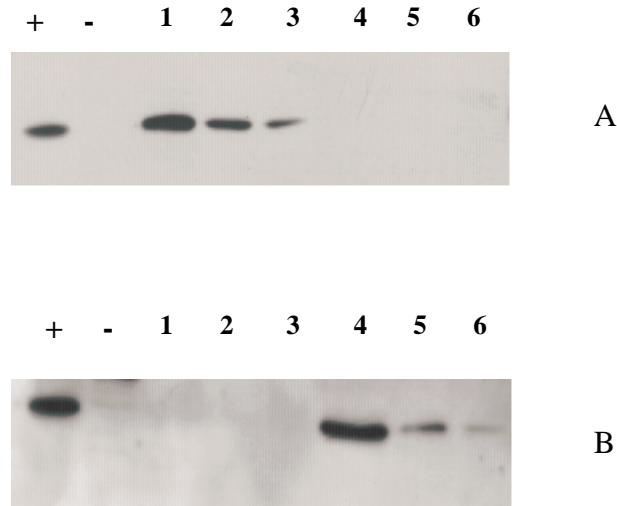


Fig. 3. M1 and NS1 cross-reactivity testing with respective antibodies.

line (+)	- MDCK cells infected with A/equi/Kentucky 81
line (-)	- MDCK mock - infected
lines 1, 2 and 3	- 50, 30 and 10 ng of purified M1
lines 4, 5 and 6	- 50, 30 and 10 ng of purified NS1
A	- reaction with antibodies against M1
B	- reaction with antibodies against NS1

Serum of guinea pig vaccinated with inactivated vaccine (HI titre of 512) was used in reaction with 30 and 50 ng of each NS1 and M1 proteins (Fig. 4 A). Strong reaction with M1 was observed. On the other hand, no reactivity was observed with NS1 protein. The same conditions were used for testing of serum of the horse experimentally inoculated with influenza virus (Fig. 4 B). Both M1 and NS1 proteins gave positive reaction. When serum of that horse before inoculation was tested (Fig. 4 C), M1 protein gave weak positive result, while NS1 antibodies were not detected.

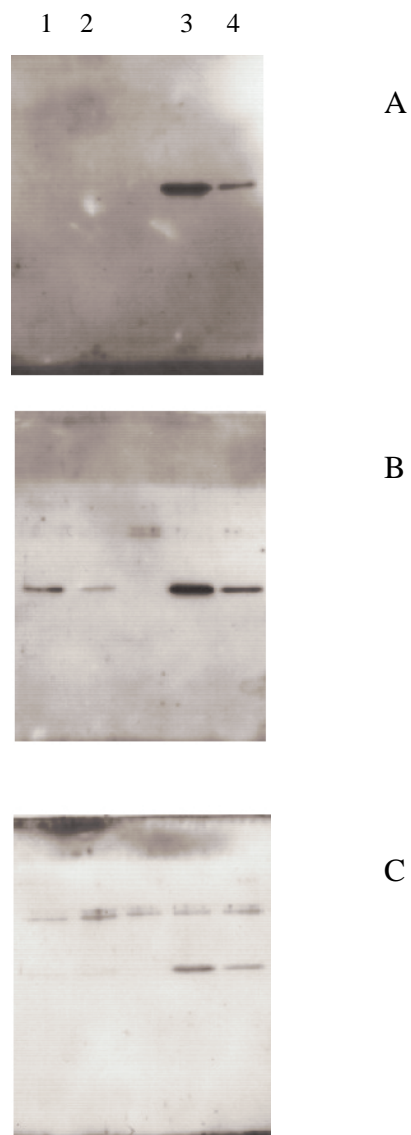


Fig. 4. Detection of antibodies for NS1 and M1 proteins in sera of animals infected and vaccinated.

1 – 50 ng of NS1

2 – 30 ng of NS1

3 – 50 ng of M1

4 – 30 ng of M1

A – serum from guinea pig vaccinated with inactivated virus

B – serum from experimentally infected horse

C – serum from experimentally infected horse, collected before inoculation

Additionally, immunoblotting with 6 randomly selected sera from horses of unknown immunological status towards influenza virus was performed (Fig. 5). Purified M1 and NS1 proteins (50 ng each) were used as antigens in immunoblot tests. In three cases (no. 3, 5, 6) positive antibody reaction with NS1 was observed which indicated a history of infection with equine influenza virus of three horses. Antibodies against M1 were also detected in those animals. Strong reaction with M1 protein and very weak signal for NS1 was detected in horses no. 1 and no. 4, which may indicate that they were vaccinated against equine influenza. Animal no. 2 showed very weak reaction against M1 protein and no reaction against NS1. This horse was not vaccinated for long time and was not infected with influenza virus.

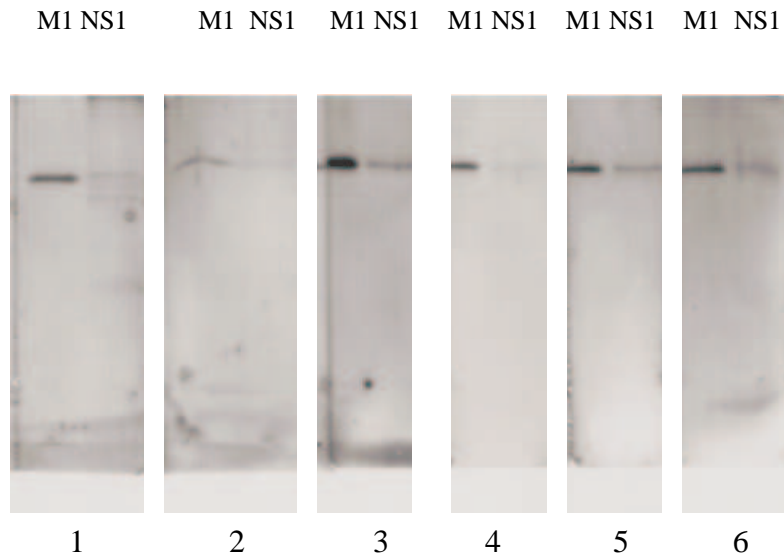


Fig. 5. Immunoblot testing of horse serum samples from the field with NS1 and M1 antigens.
1 – 6 - randomly selected serum samples from animals of unknown immunological status

Discussion

Equine influenza is one of the most important respiratory diseases in horses. Intense movement of race horses makes the transmission of the virus to susceptible animals more likely. Therefore immunoprophylaxis of equine influenza based on inactivated vaccines is a common practice. However, the effectiveness of vaccination is limited and requires frequent administration. Repeated vaccinations induce high levels of antibodies, which may complicate the detection of acute infections based on four-fold increase in HI titres in paired serum samples (1, 2, 10). Diagnostic method distinguishing between infected and vaccinated horses would be helpful in eradication

of the disease. NS1 protein is the only nonstructural protein of influenza virus and therefore inactivated vaccines do not contain NS1 antigen. Birch-Machin *et al.* (1) detected antibodies to NS1 protein in serum samples from ponies experimentally infected with influenza virus, but not in animals vaccinated with the whole inactivated virus. It was found that NS1 protein can be used as a diagnostic marker for differentiation between horses recovered from infection and vaccinated both containing high levels of antibodies. This was the first paper where antibodies to NS1 protein have been identified after influenza virus infection. Birch-Machin *et al.* (1) investigated the presence or absence of anti-NS1 antibodies from influenza virus challenged ponies by immunoblotting using recombinant NS1 antigen. Ozaki *et al.* (11) were able to distinguish in enzyme-linked immunosorbent assay, horses infected with equine H3 influenza viruses from animals vaccinated with the inactivated vaccine using recombinant NS1 antigen.

To obtain additional data on immunological status of horses in one assay, M1 protein besides NS1 was included in this study. NS1 antigen was purified from MDCK cells infected with equine influenza virus. M1 protein was purified from influenza virions. Both proteins have similar molecular weights, are not glycosylated, and both are phosphorylated. Therefore comparable immunogenicity of both proteins can be expected. Western blotting of sera from vaccinated guinea pig and experimentally infected horse was positive for M1 protein, while anti-NS1 antibodies were detected only in infected horse. Immunoblotting test with six randomly selected horse serum samples from the field was also performed. Three of them have reacted with NS1 antigen suggesting the recovery from influenza virus infection. In two cases the presence of anti-M1 antibodies in the absence of anti-NS1 antibodies, indicates that these horses have been vaccinated. Only one horse showed very weak reaction against M1 and no reactivity with NS1 protein. It may indicate that this animal was neither vaccinated nor infected recently.

A diagnostic test based on the detection of antibodies to both M1 and NS1 proteins would enable the identification of vaccinated and infected horses. Future studies will focus on practical implementation of the system described in the paper (application of purified NS1 and M1 antigens in immunoblotting with chemiluminescent detection system) for testing of field samples.

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