DETECTION OF ANTIBODIES AGAINST EQUINE INFLUENZA VIRUS BY CELL BASED ENZYME-LINKED IMMUNOSORBENT ASSAY

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Abstract

The purpose of the study was to utilise rabbit kidney cells (RK13) infected with equine influenza virus as an alternative source of viral antigens in ELISA. Peroxidase linked assay confirmed multiplication of equine influenza virus in RK13 cells. To optimise the equine serum dilutions, one negative and one positive sera (HI titer 1,280 for both antigens) were tested. The sera were examined in serial dilutions and a decrease in OD values in both cases was related to the dilution. Finally, dilution 1:160 was considered to be the optimal as the negative serum showed low level of nonspecific binding, while the positive serum gave a high OD reading. These conditions were applied to test 36 equine sera of the HI titers ranging from 20 to 2,560 and the results of cell based ELISA and HI test were compared. OD values for HI negative sera ranged from 0.025 to 0.074 for H7N7 and from 0.042 to 0.117 for H3N8. OD values for sera positive in HI test ranged between 0.058 and 1.013 for H7N7 antigen and from 0.048 to 1.01 for H3N8 antigen in cell based ELISA. The results of both tests showed correlation r = 0.785 for H7N7 and r = 0.882 for H3N8. These results proved that RK13 cells can be used in cell based ELISA to detect antibodies specific for equine influenza virus. The advantage of the cell based ELISA can also be the fact of detecting antibodies specific to NS viral proteins; however the test needs to be optimised.

Key words: equine influenza, antibodies, ELISA.

Influenza is regarded as the most important respiratory disease of horses from economical point of view (12). Influenza virus type A is an aetiological agent of the disease. There are two serotypes of influenza virus infecting horses, which are distinguished on the basis of antigenic characteristics of haemagglutinin and neuraminidase – H7N7 (type A1) and H3N8 (type A2) (18, 22). There is no evidence of circulation of A1 virus in horse population after 1980, conversely to A2 (H3N8) serotype, which constitutes an increasingly significant problem in Western Europe and the United States of America (3, 23). The diagnosis is based on the clinical signs (fever, dry deep cough, nasal discharge) and the infection is confirmed by virological examination. Virus isolation is done by inoculation of embryonated chicken eggs into the allantoic or amniotic cavity or by inoculation of the Madin–Darby canine kidney (MDCK) cell line (19, 20). The detection of virus directly in secretions may be done by PCR or by commercial diagnostic kits (5, 18). Laboratories lacking facilities for virus isolation can use serological tests. Serological diagnosis of equine influenza relies on detection of an increase in the titre of specific antibodies in paired serum samples. The haemagglutination inhibition test and the single radial haemolysis test are most frequently used in serological diagnosis of equine influenza (Office International des Epizooties, OIE, Paris, 2000: Equine Influenza; in Manual of Standards for Diagnostic Tests and Vaccines, pp. 546-557).

ELISA method has a wide range of diagnostic applications in many viral diseases (1, 15, 16). This technique enables the detection of viral antigens (antigen capture ELISA) with monoclonal or polyclonal antibodies (7, 11). Purified virions or viral proteins are mostly used as antigens in the tests used for the detection of specific antibodies in human or animal sera (4, 21).

The purpose of the study was to utilise rabbit kidney cells (RK13 cell line) infected with A1 and A2 serotypes of influenza virus as a source of viral antigens in ELISA.

Material and Methods

Viral strains The strains of equine influenza virus A/Equi/1/Prague 56 (H7N7) and A/Equi/2/Kentucky 81 (H3N8) were obtained from the National Veterinary Services Laboratories, Ames, USA. The virus was propagated in embryonated chicken eggs.
**Cell lines.** Rabbit kidney cells (RK 13 cell line) were obtained from the European Collection of Animal Cell Cultures (ECACC). The cells were propagated in Eagle’s minimal essential medium with Earle’s salts, 2 mM L-glutamine, 1% non essential amino acids, and 10% foetal bovine serum.

**Peroxidase linked assay (PLA).** RK13 cells were cultivated in 96-well plates and the monolayer was obtained within 24 h. The medium from plates was discarded and the cells were covered with allantoic fluids from chicken embryos infected with H7N7 or H3N8 viruses (HA titer 512 in both cases) diluted 1:10, 1:100, and 1:1,000 in Eagle's minimal essential medium. The inoculum was removed after 24 h post inoculation and the cells were washed once with PBS. The cells were fixed with acetone; methanol (1: 1) for 2 min at room temperature and then the plates were dried. Next, the cells were covered with 1% BSA in TBST (50 mM TRIS, 0.138 M NaCl, 0.0027 M KCl, 0.05% Tween 20, pH 8.0) at 37°C for 30 min. The monolayers were incubated with rabbit sera diluted 1: 1,000 in TBST (rabbits immunised with purified virions of H7N7, HI titre 4,128 or H3N8, HI titre 8,024) at 37°C for 30 min. After four washes with TBST, the monolayers were incubated with conjugate solution [1: 1,000 of (Fab')2 fragments of goat IgG anti Fc fragments of rabbit IgG - horseradish peroxidase (Jackson Immunoresearch) in TBST] at 37°C for 30 min. After four washes with TBST, substrate for peroxidase was used. After 20 min incubation at room temperature reaction was stopped by washing with distilled water and the plates were dried out. The results were analysed under microscope at magnification of 40x.

**Haemagglutination and haemagglutination inhibition assays (HA, HI).** HA and HI assays were performed according to the standard OIE procedures (OIE, Paris, 2000, Equine Influenza; in Manual of Standards for Diagnostic Tests and Vaccines, pp. 546-557).

**Cell based ELISA.** RK13 cells in 96-well plates, infected with H7N7 or H3N8 viruses were fixed and incubated with BSA as described above and reacted with two equine sera: HI positive (the titre 1,280 for both influenza serotypes) or HI negative. The sera were serially diluted in 1% BSA/TBST - 1: 10, 1: 40, 1: 160, 1: 640, 1: 1,280, 1: 2,560, and 1: 10,240. Each serum dilution was applied to four wells (sample wells) and to four wells in the column were filled with 1% BSA/TBST (control wells). Plates were incubated at 37°C for 60 min. After four washes with TBST, monolayers were incubated with conjugate solution [1: 1,000 of (Fab')2 fragments of goat IgG anti Fc fragments of rabbit IgG - horseradish peroxidase in TBST] at 37°C for 30 min. After four washes with TBST, TMB substrate was applied (FastDAB™ - Sigma-Aldrich). After 20 min incubation at room temperature, the reaction was stopped with 1N HCl and the signal was detected at 450 nm in the microplate reader (Synergy HT™, BioTek, USA). OD values for each serum sample were calculated by subtracting the mean OD of four control wells from the mean OD of four sample wells. Thirty-six field equine sera were selected based on HI assay results. They were divided into three groups: A - sera with negative HI, B - sera with HI titres 10-160, and C - sera with titres 640-2,560 for both equine influenza virus subtypes. The whole cell based ELISA procedure was carried out as described above. Sera were applied at the single working dilution of 1:160.

**Results**

Peroxidase linked assay confirmed multiplication of equine influenza virus in RK 13 cells (Fig. 1). The H7N7 HA 512 allantoic fluid diluted 1:100 and the H3N8 HA 512 allantoic fluid diluted 1:1,000 caused comparable levels of infection with the limited cytopathic effect. H7N7 serotype infected only single cells at 1:1,000 dilution while cytopathic effect was observed at 1:10 dilution. Infection with H3N8 serotype was more intense, and cytopathic effect was observed at 1:10 and 1:100 dilutions. Thus, the 1:100 dilution of H7N7 and the 1:1,000 dilution of H3N8 were used as the inoculum of RK13 in further experiments.

To optimise the ELISA, the equine serum dilutions, one negative and one positive serum (HI titre 1,280 for both antigens), were tested. The correlation of OD values and serum dilutions is presented in Fig. 2 and Table 1. OD values for negative serum at 1:10 dilution were similar for both antigens (0.968 for A1 and 1.083 for A2). A decrease in OD values was observed along with the serial dilutions of negative sera, which suggested a reduction of non-specific binding. OD value for positive serum was 2.313 for H7N7 and 2.446 for H3N8 at 1:10 dilution, and its value decreased with the consecutive dilutions (r=0.9927 for H7N7 and r=0.9572 for H3N8). The dilution 1:160 of the positive sera was considered to be the optimal for further experiments (a negative serum showed low level of non-specific binding, while specific signal for positive serum was high).

The dilution 1:160 was applied to test 36 field equine sera ranging in HI titers from 20 to 2,560 for A1 and A2 antigens. OD values for HI negative sera ranged from 0.025 to 0.074 for H7N7 and from 0.042 to 0.117 for H3N8. The mean OD value for these samples was 0.0532 (SD 0.0153) for H7N7 antigen and 0.0811 (SD 0.0229) for H3N8 antigens. OD values for sera positive in HI test ranged between 0.058 and 1.013 for H7N7 antigen and from 0.048 to 1.01 for H3N8 antigen in cell based ELISA. The correlation factor of ELISA and HI test results was r=0.785 for H7N7 and r=0.882 for H3N8. The cut off value for positive sera was calculated as a sum of mean OD value for negative samples and two standard deviations (OD neg. + 2 SD), it was 0.0838 and 0.127 for H7N7 and H3N8, respectively. Some OD values for weak positive sera (HI titer 40 or less) were below the cut off value calculated as described above (Figs 3 and 4).
Fig. 1. Immunoperoxidase staining of RK13 cells infected with equine influenza virus. N.I. – non-infected culture; A1 - cells infected with A/equi/Prague 56; A2 - cells infected with A/equi/Kentucky 81; 10, 100, and 1,000 - dilutions of allantoic fluids from infected chicken embryos used for culture inoculation. FastDAB™ substrate for peroxidase was used.

Table 1

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>Antigen A1</th>
<th>Antigen A2</th>
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<tbody>
<tr>
<td></td>
<td>Negative serum</td>
<td>Positive serum</td>
</tr>
<tr>
<td>1: 10</td>
<td>0.968</td>
<td>2.313</td>
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<tr>
<td>1: 40</td>
<td>0.312</td>
<td>1.741</td>
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<tr>
<td>1: 160</td>
<td>0.088</td>
<td>1.436</td>
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<tr>
<td>1: 640</td>
<td>0.063</td>
<td>1.045</td>
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<tr>
<td>1: 1,280</td>
<td>0.052</td>
<td>0.973</td>
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<tr>
<td>1: 2,560</td>
<td>0.029</td>
<td>0.531</td>
</tr>
<tr>
<td>1: 10,240</td>
<td>0.012</td>
<td>0.191</td>
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</tbody>
</table>

Fig. 2. Cell based ELISA of HI negative or positive horse sera. RK13 cells infected with A/equi/Prague 56 (antigen A1) or A/equi/Kentucky 81 (antigen A2) were used as a solid phase. The serum dilution factor of 160 (indicated by arrows) was chosen for further experiments.
Fig. 3. Cell based ELISA of 36 field horse sera: negative, weak positive (HI 10-160) or strong positive (640-2,560) in HI assay. Threshold lines indicated the level calculated as a sum of mean OD value for negative samples and two standard deviations (OD neg. + 2 SD).

Fig. 4. The distribution of OD values for groups of negative - A, weak positive (HI 10-160) - B or strong positive (640-2,560) in HI assay - C. Horizontal bars indicate the mean OD value for each group.

Based on this calculations it has been noted that some HI positive sera for A1 (HI 20, 40, 80, and 160) gave positive readings in ELISA but some others gave the negative results in ELISA (Fig. 3 B, Fig. 4). The same observation relates to the sera positive for A2 (Figs 3 B, 4). The sera with high HI titers for A1 and A2 gave constant high readings in cell based ELISA (Figs 3 C, 4); however, it was noted that among them some sera with the lower HI titers (e.g. 640) gave higher OD values in the cell based ELISA then the sera with higher HI titers (e.g. 1,280), (A1 Fig. 3 C). Some A2 positive sera gave comparable OD readings irrespective of HI titers (A2 Fig. 3 C).
Discussion

ELISA can be used to detect specific antibodies in diagnosis of viral diseases (13, 15). Complete virions or purified viral proteins are most frequently used in these types of assays (4, 6, 21). Application of infected cells without additional treatment as a source of antigens, used as a solid phase in ELISA could be an alternative procedure. Many different cell lines are susceptible to influenza A virus infection. MDCK cells are frequently used for multiplication of virus during vaccine production and they may serve as an alternative to embryonated chicken eggs in virus isolation (8, 14, 17). Cell cultures also may be used to monitor virus susceptibility to antivirals. The optimised in situ cell based ELISA served as a reliable assay for rapid screening of large number of antiviral agents (9).

MDCK cells are more susceptible to infection with H3N8 than with H7N7 serotype (20). It is difficult to obtain comparable levels of infection even with addition of trypsin, which stimulates proteolytic activation of haemagglutinin. However, similar level of cell infection with both equine influenza virus subtypes is crucial for reliable estimation of antibody levels in equine sera. An attempt was made to implement the RK13 cell line, which is more uniform in terms of susceptibility to infection and cytopathic effect developing for both serotypes of influenza virus.

The first step was to evaluate the susceptibility of RK13 cells to equine influenza virus infection. The choice of the optimal viral doses allowed getting comparable level of infection for both strains with limited cytopathic effect. The next step was to test one HI positive and one HI negative equine serum in cell based ELISA. Sera were examined in serial dilutions and a decrease in OD values in both cases was related to the dilution. Finally, dilution 1:160 was considered to be the optimal as the negative serum showed low level of non-specific binding, while the positive serum gave a high OD reading. These conditions were applied to test 36 field equine sera and the results of cell based ELISA and HI tests were compared. The results of both tests showed correlation r=0.785 for H7N7 and r=0.882 for H3N8. These results proved that RK13 cells can be used in cell based ELISA to detect antibodies specific for equine influenza virus; however, the test needs to be optimized. More accurate determination of cut-off value would be possible after examination of larger number of negative and HI low titer samples and with possible pretreatment of sera to reduce non-specific binding to the cell cultures.

Theoretically, cell based ELISA may be useful for differentiation of infected and vaccinated animals. Infected cells contain full set of viral antigens (structural and nonstructural proteins). Vaccines for equine influenza contain inactivated virions. Therefore, immunological response after vaccination is limited to the structural proteins only. As a result of the natural infection antibodies for nonstructural protein NS1 are produced (2, 10). Cell fixation procedure used in our study, enables accessibility to the intracellular antigens (permeabilisation of cell membranes). Therefore, a part of signal would be derived also from NS1 protein. Although the number of copies of M2 protein in virions is low, the expression in cell membranes during infection is high (24). Therefore, the signal from the sera of vaccinated and naturally infected horses, obtained in the cell based ELISA for the sera with the same HI titers, should give stronger reaction in infected horses. The results obtained for the HI and ELISA test in the range of low HI titers (10-160), as well as different OD readings in ELISA for the sera with high HI titers (640-2,580) gives some assumption for this suggestion.

The introduction of cell based ELISA to serological diagnosis of infection with equine influenza virus seems to be a promising alternative to HI assay. ELISA has many advantages like: simplicity of procedure, possible automation, or lack of human error during results reading. Moreover, preparation in advance of large number of ready-to-use plates is possible. Both biological and technical features of cell based ELISA presented in this study points to the possible use of this method in the diagnosis of equine influenza infections.

References


