# EVALUATION OF CLINICAL SPECIMENS FOR INFLUENZA A VIRUS POSITIVITY USING VARIOUS DIAGNOSTIC METHODS

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**Summary. –** The diagnostic method for Influenza A virus, utilizing the SERION ELISA Antigen kit (SERION EIA), if results were evaluated according to the manufacturer's instructions, has repeatedly failed to detect a great number of clinical samples positive by virus isolation and RT-PCR. Therefore we compared the SERION EIA with the one-step 44/107L-Px immunocapture enzyme immunoassay (44/107L-Px EIA), developed in our laboratory (Tkáčová and Varečková, *J. Virol. Methods* **60**, 65–71, 1996). Seventy-three clinical specimens, of which 65 were positive by virus isolation (used as reference method), were tested by both EIAs. By the SERION EIA, out of the 65 reference-positive samples only 8 (12%) were positive, 5 (8%) were ambiguous, and 52 (80%) were negative, which corresponded to the sensitivity of 12%. On the contrary, the sensitivity of the 44/107L-Px EIA was 74%. However, the calculation of cut-off values for the evaluation of positivity of clinical specimens in these two assays were not the same. If the evaluation procedure used for the 44/107L-Px EIA was applied to the SERION EIA, the sensitivity and the specificity of both EIAs became comparable, namely 71% and 100% for the SERION EIA and 74% and 100% for the 44/107L-Px EIA, respectively. From these results it follows that not the detection ability of the SERION EIA, but the evaluation procedure recommended by its manufacturer led to a loss of large number of positive specimens.

**Key words:** Influenza A virus; clinical specimens; diagnostics; enzyme immunoassay; SERION ELISA Antigen kit; sensitivity, specificity; virus isolation; RT-PCR

## Introduction

Influenza A viruses cause an acute respiratory disease that spreads epidemically in the human population every year (Cox *et al*., 1996). The reason of repeated influenza A infections is the high variability of the respective viruses, preferentially their surface antigens: hemagglutinin and neuraminidase (Webster and Laver, 1975; Both *et al*., 1983; Skehel and Wiley, 2000) . The emerging of fatal cases of human infections with avian influenza viruses of H5 subtype (Webster, 1997; Subbarao *et al*., 1998; Peiris *et al*., 1999; Lin *et al*., 2000; Horimoto and Kawaoka, 2001; Guan *et al*., 2004) that are considered potentially pandemic led the scientists to search for the way to prevent this threat. Despite great efforts no universal influenza vaccine has been developed yet (Palese and Garcia-Sastre, 2002). Therefore the vaccine programs based on careful monitoring of epidemic strains and characterization of their antigenic structure and gene sequences have been elaborated (Cox *et al*., 1994; Nicholson 2000; Laver and Garman, 2001). They enable the actualization of vaccine strains every year. Simultaneously, novel antiviral drugs, which may temporarily prevent the spread of infection, are being tested. For the monitoring of influenza A infections, from prophylactic or therapeutic reasons, various diagnostic methods have been developed (Rebelo de Andrade and

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**Abbreviations:** EIA = enzyme immunoassay, SERION EIA = EIA using the SERION ELISA Antigen kit; MAb = monoclonal antibody; NP = nucleoprotein; 44/107L-Px EIA = one-step 44/107L-Px immunocapture EIA

Zambon 2000; Linde, 2001). A standard method that is used in specialized laboratories is virus isolation, i.e. the virus propagation in tissue culture or embryonated henn's eggs. The isolated virus is then characterized antigenically and by gene sequencing. Recently, a faster modification, the rapid culture assay (RCA) was developed; it enables identification (typing and subtyping) of influenza virus isolates in clinical samples already after 18 hrs of cultivation in monolayer cell cultures using specific monoclonal antibodies (MAbs) (Waris *et al*., 1990a; Ziegler *et al*., 1995; Tkáčová *et al*., 1997; Varečková *et al*., 2002). All these procedures enable isolation of the virus from clinical samples within the time ranging from overnight (RCA) to several days (virus isolation). They are not suitable for early influenza diagnosis needed for effective chemotherapy. For this purpose, less time-consuming methods able to detect the viral antigen directly in clinical samples, have been developed. These are various modifications of the immunoassay: EIA, time-resolved fluoro-immunoassay radioimmunoassay, etc. (e.g.Walls *et al*., 1986; Waris *et al*., 1990b; Tkáčová *et al*., 1996; Varečková *et al*., 2001). Their advantage is the rapidity (1–2 hrs), though their sensitivity is limited and depends on the quality and epitope specificity of antibodies used in the assay (Jackson and Ekins, 1986). Their sensitivity and specificity are 40–99% and 86–99%, respectively. To these methods belongs also a novel multiplex microsphere-based immunoassay read by flow cytometry (Yan *et al*., 2004). It is highly sensitive and enables to analyze a large number of samples and detect more than one antigen by one assay

There are available various kits suitable for influenza A diagnostics directly in clinical specimens. One of them, used at The National Influenza Centre, Public Health Authority of the Slovak Republic, is the SERION ELISA Antigen kit. As a relative low positivity of clinical specimens was observed with SERION EIA assay, we compared it with the 44/107L-Px EIA, the one-step-immunocapture assay developed in our laboratory (Tkáčová and Varečková, 1996) based on the capture MAb 44 and the detector MAb 107L conjugated to horseradish peroxidase. Virus isolation and RT-PCR were used as reference and confirmation methods, respectively.

## Materials and Methods

*Clinical specimens.* Nasopharyngeal swabs were collected during the epidemic season 2003/2004 in a transport medium and stored at -20°C. To prepare samples for both EIAs, the nasopharyngeal swabs were diluted 1:1 with a specimen buffer (a part of the SERION ELISA Antigen kit) and incubated for 1 hr at 37°C.

*44/107L-Px EIA* was carried out as described previously (Tkáčová and Varečková, 1996; Varečková *et al*., 2001). To enable the capture of the Influenza virus NP from specimens, microplate wells were coated with the MAb 44 (250 ng/100 µl/well) overnight at 4°C, washed with PBS and saturated with 1% non-fat dry milk for 1 hr at room temperature. After washing with the EIA buffer (0.154 mol/l NaCl, 0.1 mol/l Tris-HCl pH 7.2, 0.1% non-fat dry milk, and 0.01% Tween 20), specimen (50 µl/well) and the detector MAb 107L conjugated with horseradish peroxidase (80 ng/50 µl/well) were added, then the microplates were incubated at room temperature for 1 hr. The unbound antigen and the detector MAb were removed by washing with the EIA buffer and an activated substrate solution (0.5 mg/ml orthophenylenediamine (Sigma) in McIlvaine's buffer pH 5.0 containing 0.03% hydrogen peroxide) (100 µl/well) was added. The colour reaction was developed for 15 mins in the dark and was terminated by adding 3 N HCl (100 µl/well). $A_{492}$ was read in a Multiscan spectrophotometer (MCC/340, Labsystems, Finland). The cut-off value was calculated as average $A_{492}$ of negative controls plus 3 SD, i.e. 0.052.

*MAbs* 44 and 107L, recognizing two distinct non-overlapping epitopes on the viral NP, were emloyed (Varečková *et al*., 1995). The purified detector MAb 107L was conjugated with the Type VI-A horseradish peroxidase (Sigma) according to Wilson and Nakane (1978). The molar ratio of peroxidase/IgG was 1.42 ($A_{403}/A_{280} = 0.398$).

*SERION EIA* for direct detection of Influenza A virus antigen in clinical specimens was performed using the SERION ELISA Antigen kit according to the manufacturer's instructions (Institute Virion/Serion GmbH, Germany). The samples were added to microplates coated with an antibody to Influenza A virus (the capture antibody) (100 µl/well). After incubation at 37°C for 1 hr in a humid chamber the wells were washed 4 times and a detector antibody conjugated with horseradish peroxidase was added (100 µl/well). After 30 mins at 37°C the unbound detector antibody was removed by washing and a substrate solution containing tetramethylbenzidine and hydrogen peroxide was added (100 µl/well). After 20 mins at room temperature in the dark the reaction was terminated with a stop solution, and $A_{450}$ was read in a microplate reader. The calculation of positivity of samples recommended by the manufacturer of the respective kit was as follows. The cut-off value for positive samples was estimated as average $A_{450}$ of negative controls (0.003) plus 0.250, i.e. 0.253, and the cut-off value for negative samples was estimates as average $A_{450}$ of negative controls (0.003) plus 0.150, i.e. 0.153. Thus a sample with $A_{450}>0.253$ was considered positive, that with $A_{450}<0.153$ negative, and that with $A_{450} = 0.153–0.253$ ambiguous.

*Virus isolation*. MDCK cells were grown to monolayer in tubes in Dulbecco's Minimum Eagle's Medium (DMEM) containing 5% FCS (the cultivation medium) in a standard manner. The cultures were washed twice with DMEM and nasopharyngeal swabs diluted 1:1 with the cultivation medium were added (0.2 ml/culture). After 30 mins of incubation at room temperature, the cultivation medium containing 3 µg/ml TPCK trypsin (Sigma) was added (2 ml/culture) and the incubation continued at 34°C. Cytopathic effect on the cells and hemagglutination activity of the medium were followed daily. The samples with a significant hemagglutination titer were characterized for the virus type/subtype in a hemagglutination-inhibition (HI) test.

*Hemagglutination titration* of culture supernatant fluids was done using their serial 2-fold dilutions (50 µl/well) and 0.75% human „0“ red cells in PBS (50 µl/well) in 96-well round-bottom microtiter plates in standard manner.

*HI titration* was carried out in standard manner using specific ferret immune sera against influenza viruses A /NewCaledonia/20/99 (H1N1), A/Fujian/411/02 (H3N2), and B/Sichuan/379/99 (all provided by WHO Collaborating Centre for Reference and Research on Influenza, NIMR, London, UK). The virus was identified as of particular type/subtype if it gave with one antiserum at least 4 times higher titer than with others.

*Total RNA* extraction and purification from samples (100 µl), based on standard phenol-chloroform method, was done with RNA Insta-Pure System (EUROGENTEC, Belgium) (500 µl) according to the manufacturer's instructions. The purified RNA was dissolved in 20 µl of water containing 2 U/µl RNase inhibitor (Fermentas).

*RT-PCR* amplified a 510 bp fragment of the viral NP gene. The RT reaction mixture (20 µl) consisted of 5 µl of total RNA, 1 mmol/l dNTPs, 0.2 µg of a random heptamer (Invitrogen), 200 U of MMLV reverse transcriptase (Fermentas) in 1x RT buffer (Fermentas). The reaction ran for 60 mins at 42°C. In the PCR step, the reaction mixture (20 µl) contained 10 pmoles of each of forward (5'-GTGAGGATGCAACAGCTGGTCTAAC-3') and reverse primer (5'-TACCCCTCTTTTTCGAAGTCGTAC-3'), 2 µl of cDNA, and 1x PCR Master Mix (Fermentas). The primers for the NP gene of the virus of H1 and H3 subtype were selected using the Primer3 software (SAS EMBnet node, EMBnet Slovakia, URL: http://www.embnet.sk/). Particular virus strains were chosen from the database (URL: http://www.flu.lanl.gov/, Acc. No. AB12664 (NP 377-400, 886-864)). PCR cycling conditions consisted of 95°C/60 secs, 35 cycles of 95°C/30 secs (denaturation), 55°C/30 secs (annealing), and 72°C/45 secs (elongation). The PCR products (5–10 µl/lane) were detected by 2% agarose gel electrophoresis (110 V, 20 mins) in the presence of ethidium bromide. The PCR was able to detect 1.12 pg of cDNA.

## Results

*Influenza A virus positivity of clinical specimens determined by virus isolation and RT-PCR*

Out of 73 clinical specimens 65 positive and 8 negative were found by virus isolation. All the specimens positive by virus isolation were also positive by RT-PCR, and the same was valid for negative specimens (Table 1).

*Evaluation of specificity and sensitivity of SERION-EIA and 44/107L-Px EIA*

The ability to detect the viral antigen in clinical specimens was examined by SERION EIA and 44/107L-Px EIA.

SERION EIA, with the evaluation of positivity as recommended by the manufacturer of the respective kit, revealed in 65 clinical specimens (positive by virus isolation and RT-PCR) 8 positive, 5 ambiguous and 52 negative (Table 1). This result corresponded to a sensitivity of 12% (8/65). A much higher positivity was obtained with the same specimens by 44/107L-Px EIA, namely 48 positive and 25 negative specimens, which represented a sensitivity of 74% (48/65). However, it should be emphasized that the evaluation of positivity in this assay differed markedly from that of SERION EIA. In particular, the difference resided in the cut-off values (see *Materials and Methods*).

Therefore, we reevaluated the positivity of SERION EIA by applying the calculation procedure used in 44/107L-Px EIA, namely the cut-off value defined as the mean absorbancy of negative controls plus 3 SD. In this way, with a cut-off value of 0.011, a much higher positivity as compared to the original was obtained: 46 positive and 27 negative specimens, corresponding to a sensitivity of 71% (46/65) (Table 1).

All the specimens negative by both EIAs were also negative by virus isolation and RT-PCR, i.e. the specificity of both EIAs was 100%.

Evaluation of two distinct groups of clinical specimens from the epidemic season 2003/2004 for the virus positivity is given in Fig. 1. In both groups (a, b) the specimens positive by virus isolation exhibited a linear relationship between absorbancy values in two compared assays, reflected by the Pearson's correlation coefficient close to 1.00 (0.99 and 0.97 respectively).

**Table 1. Detection of Influenza A virus in clinical specimens by 44/107L-Px EIA and SERION EIA**

| Virus isolation[a] | | PCR[b] | | SERION[c] | | | SERION[d] | | EIA 44/107L-Px[d] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | + | – | + | ± | – | + | – | + | – |
| + | 65 | 63 | 0 | 8 | 5 | 52 | 46 | 19 | 48 | 17 |
| – | 8 | 0 | 8 | 0 | 0 | 8 | 0 | 8 | 0 | 8 |
| | 73 | 63 | 8 | 8 | 5 | 60 | 46 | 27 | 48 | 25 |

[a]Reference method.
[b]Confirmation method. Two clinical specimens were not tested.
[c]Cut-off value calculated according to the manufacturer's instructions of the kit employed.
[d]Cut-off value calculated as average absorbancy of negative controls plus 3 SD.
± Specimens with borderline values.

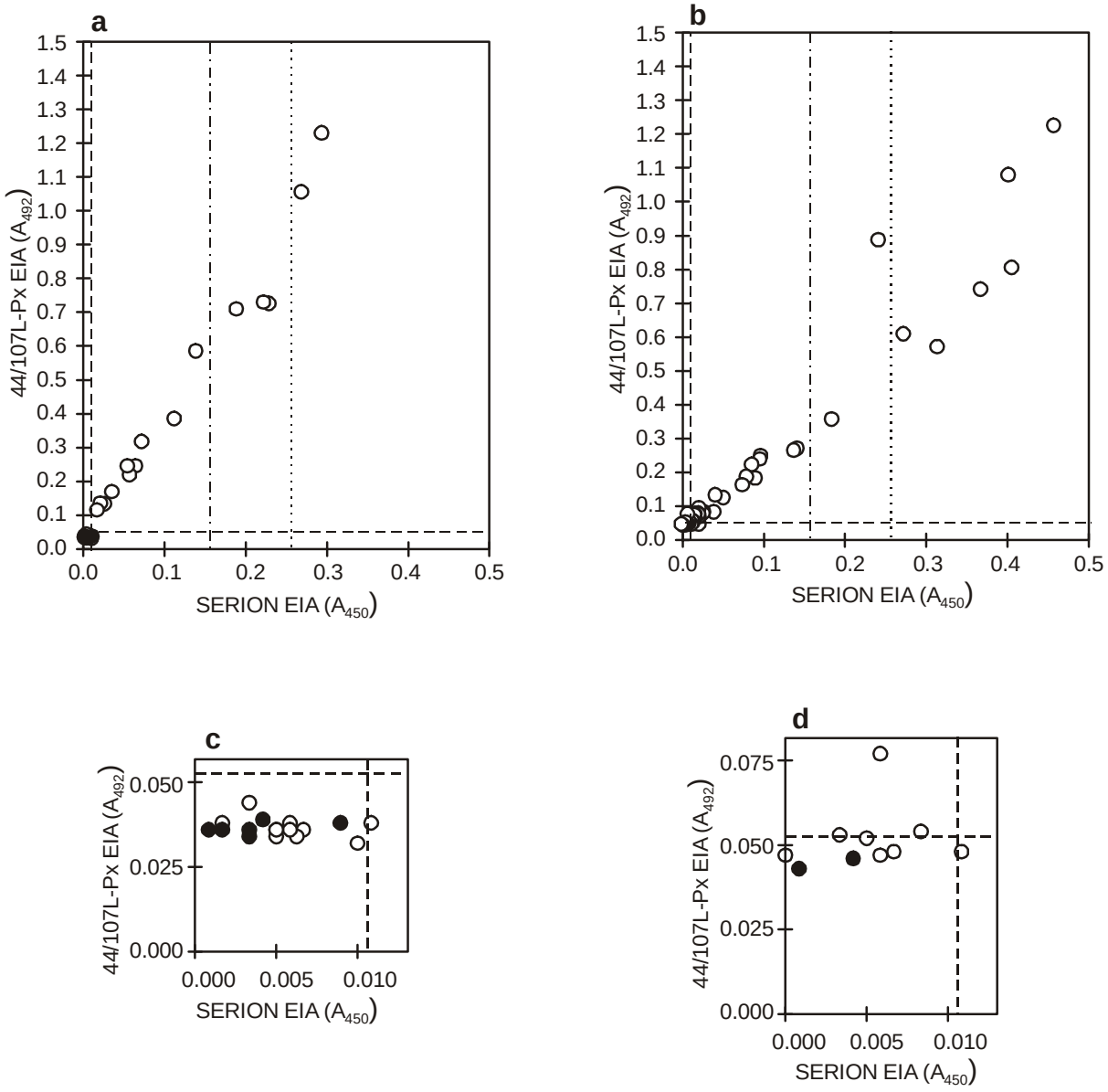


**Fig. 1**

**Detection of Influenza A virus in clinical specimens by SERION EIA and 44/107L-Px EIA**

First (a) and second (b) group of specimens and detailed view of negative specimens (c) and (d), taken from (a) and (b) respectively. Specimens positive (empty circles) and negative (full circles) by virus isolation. Cut-off value defined as average absorbancy of negative controls plus 3 SD as recommended for 44/107L-Px EIA (- - - -). Cut-off value for negativity as recommended for SERION EIA (- · - · ). Cut-off value for positivity as recommended for SERION EIA (· · · ·). SERION EIA borderline values range between negative and positive cut-off lines.

The linear regression coefficient between absorbance values in the two assays was 2.69 and 2.28 in the first (a) and second group (b) of clinical specimens.

## Discussion

Effective antiviral chemotherapy and immediate monitoring of potentially highly pathogenic Influenza A viruses require rapid and very sensitive diagnostic methods able to detect the viral antigen directly in clinical specimens.

SERION EIA is currently used in the field diagnostics of influenza viruses at the National Influenza Centre, Public Health Authority of the Slovak Republic, Bratislava. In this assay, the positivity of specimens for presence of the virus is evaluated according the instructions of the manufacturer of the respective kit. Because this method has repeatedly

failed to detect the virus antigen in a great number of specimens positive by virus isolation, we compared its results with those of 44/107L-Px EIA. The latter, developed in our laboratory (Tkáčová and Varečková, 1996), is based on a pair of MAbs recognizing two non-overlapping epitopes on the viral NP. Its detection limit was previously determined at 10 pg/100 µl of purified influenza A virus NP, representing the highest sensitivity so far obtained by an immunoassay (Walls *et al*., 1986; Varečková *et al*., 2001).

Both SERION EIA and 44/107L-Px EIA are of immunocapture type, i.e. the antibody specific to the viral NP is used as a captor, and another, specific to the NP conjugated with horseradish peroxidase, is used as a detector. However, while 44/107L-Px EIA is an one-step assay, i.e. a sample and the detector antibody are added simultaneously, SERION EIA is a two-step assay: first a sample is incubated with the capture antibody, the unbound antigen is removed and the detector antibody is added. The two-step SERION EIA is thus more time-consuming than the one-step 44/107L-Px EIA. Moreover, 44/107L-Px EIA requires only one half of the volume of sample (50 µl/well) as compared to the SERION EIA (100 µl/well).

For the purpose of appropriate comparison of both EIAs we tested 65 clinical specimens with the positivity assessed by virus isolation in cell culture (infectious virus) and confirmed by RT-PCR (viral RNA). The selected specimens were very weakly but significantly positive by both assays. Such a selection enabled us to distinguish the sensitivity of the EIAs very precisely.

The specificity of both EIAs was 100%. However, their sensitivity changed in dependence on the cut-off values employed. If the cut-off value for positivity was set equally for both EIAs (mean absorbancy of negative controls plus 3 SD), their sensitivities were comparable. In fact, the sensitivity of 44/107L-Px EIA was slightly higher (74%) than that of SERION EIA (71%). The slight difference could be caused by a high affinity of MAbs 44 and 107L ($K = 7.8 \times 10^8$ l/mol and $K = 1.4 \times 10^9$ l/mol respectively) (Varečková *et al*., 1995). However, using the calculation of the cut-off value recommended by the producer of the SERION EIA kit, many positive specimens became negative or ambiguous. Consequently, the sensitivity of SERION EIA depending on the cut-off value for positivity decreased from 71% (with the cut-off value equal to the mean absorbancy of negative controls plus 3 SD) to 12% (with the cut-off value equal to the mean absorbancy of negative controls plus 0.250). From these data it follows that the sensitivity of the compared EIAs depends on the way of their evaluation. Also, the proportion of false-negative specimens is markedly influenced by the setting of the cut-off value for positivity. In using an EIA for Influenza A virus it should be considered for which purpose it is performed.

In any case, EIAs still remain valuable diagnostic tools for rapid detection of influenza A infection by the physician of first contact, when an immediate diagnosis is needed. In case of severe clinical symptoms it may be supposed that a high level of viral antigen is present in the given clinical specimen, and thus EIA can be used as a reliable diagnostic method. However, in case of mild infection, the low level of viral antigen in the clinical specimen might not be detectable and a false-negativity could occur. In this case, more precise methods should be used. Therefore, EIA cannot substitute virus isolation or RT-PCR, which are more laborious but most reliable methods suitable for a very sensitive detection of influenza A infection as well as monitoring of respective epidemic virus strains.

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