

DIAGNOSTIC POTENTIAL OF THE HAEMAGGLUTINATION  
INHIBITION TEST, THE IMMUNODIFFUSION TEST AND ELISA  
FOR DETECTION OF ANTIBODIES IN CHICKENS,  
INTRAVENOUSLY INFECTED WITH A/DUCK/BULGARIA/05  
H6N2 AVIAN INFLUENZA VIRUS ISOLATE

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**Summary**

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After experimental infection of chickens with an avian influenza viral isolate A/duck/Bulgaria/05 H6N2, the potential of the haemagglutination inhibition test (HIT), the immunodiffusion test (IDT) and enzyme-linked immunosorbent assay (ELISA) for antibody detection was evaluated. The results evidenced that in birds, haemagglutinins, precipitins and IgG antibodies, detectable with ELISA, were formed. The percentage of chickens with subtype-specific antibodies was the highest by the 21<sup>st</sup> day of infection (100 %), followed by the 14<sup>th</sup> (66.7 %) and the 28<sup>th</sup> (55.6 %) days, and was the lowest by the 7<sup>th</sup> day (44.4 %). Serum titres ranged between 1:4 and 1:256 with predomination of 1:8 and 1:16 titres (29.2 % each). The mean arithmetic titre for the experiment was 1:38.2. The highest percentage of chickens with precipitins was observed by the 14<sup>th</sup> day (55.6 %) followed by the 7<sup>th</sup>, 21<sup>st</sup> and the 28<sup>th</sup> days with 33.3 % each. The titres ranged from undiluted to 1:4 with prevailing of 1:2 titres. By means of ELISA, 55.6% seropositive birds were detected by the 14<sup>th</sup> day whereas the percentage on days 7, 21 and 28 was 33.3%. S/P values were the highest by the 14<sup>th</sup> day (up to 1.640). The sensitivity was 80.6 % for HIT and 46.7 % for both IDT and ELISA on the background of a specificity of 100 %. The comparison of the three tests showed a specificity of 98.2 % for IDT and ELISA against HIT, sensitivity of 54.2 % for each of IDT and ELISA vs HIT and test agreement of 84.6 % for each of IDT and ELISA vs HIT. The data for detection of type-specific antigens showed equal results for both IDT and ELISA – specificity of 98.4 %, sensitivity of 92.9 % and test coverage of 97.4 %.

**Key words:** avian influenza viruses, chickens, ELISA, haemagglutination inhibition test, immunodiffusion test

INTRODUCTION

After the penetration of an avian influenza virus (AIV), the organism reacts with antibody formation. They are formed both against the superficial viral antigens (haemagglutinins, neuraminidase) as well as against the deeper ones (matrix, nucleo-

proteid). For their detection, a number of diagnostic tests have been developed. Most commonly, antibody detection is performed by means of the haemagglutination inhibition test (HIT), the immunodiffusion test (IDT) and ELISA.

In the reports of many authors, there is considerable variation in data for comparative studies upon diagnostic tests for detection of respective antibodies in spontaneous (Adair *et al.*, 1989; Adair *et al.*, 1990; Arenas *et al.*, 1990; Boer *et al.*, 1990; Lamichhane & Kirkegaard, 1997; Zhou *et al.*, 1998; Sala *et al.*, 2003; Meilin *et al.*, 2004) and experimental (Meulemans *et al.*, 1987; Beck & Swayne, 1997) infections. In some cases, higher positive results have been obtained in sera tested by means of HIT compared to either IDT or ELISA (Zhou *et al.*, 1998; Sala *et al.*, 2003). In other studies, ELISA was found out to provide better results than HIT or IDT (Adair *et al.*, 1989; Adair *et al.*, 1990; Arenas *et al.*, 1990; Boer *et al.*, 1990), whereas in third – no significant differences among tests have been observed (Meulemans, 1987; Beck & Swayne, 1997). The experiments of Zhou *et al.* (1998) established a sensitivity of 90.3 %, specificity of 100 % and test coverage of 95.8 % for ELISA with regard to HIT. The respective values of IDT against ELISA were 100 %; 98.2 %; 99.9 %.

The experiments about the dynamics of positive results also showed various data. Beck & Swayne (1997) detected a maximum percentage IDT-positive sera at the beginning of the infection (5<sup>th</sup> day), followed by ELISA (53.8 %) and HIT (7.7 %). Meulemans *et al.* (1987) found out HIT to be advantageous (positive from the 4<sup>th</sup> post infection day onward – 33 %) than both ELISA (positive from the 6<sup>th</sup> post infection day onward – 11 %) and IDT (positive from the 11<sup>th</sup> post infection day onward – 43 %). In both experiments, at a later period (post infection days 10 and 11), the results of these tests were similar. Haemagglutinins' titres in the beginning were low (1:2–1:4), after that

they increased up to 1:512–1:1024 (Meulemans *et al.*, 1987).

The purpose of the present study was to investigate the diagnostic potential of standard HIT and ELISA protocols as well as of a concentrated live antigen for IDT after experimental generalized infection (intravenous infection) of chickens with A/duck/Bulgaria/05 H6N2 AIV isolate.

## MATERIALS AND METHODS

### *Virus*

In this experiment, an AIV A/duck/ Bulgaria/05 H6N2 isolate from a wild duck (*Anas platyrhynchos*) was used (Zarkov *et al.*, 2006). The virus was titrated for detection of embryonic lethal dose (ELD<sub>50</sub>) by the method of Reed & Muench (1938). A viral suspension from the 4<sup>th</sup> passage with a titre of 10<sup>5.0</sup> ELD<sub>50</sub>/0.1 mL was used.

### *Experimental chickens*

The experiment was performed with 18 30-days old Dekalb chickens. Nine of them were intravenously infected with 0.1 mL allantoic fluid of virus-infected chick embryos (CE) and the other nine remained uninfected (controls) and were intravenously treated with allantoic fluid of uninfected CE.

The chickens were housed in two isolated premises of 2 m<sup>2</sup> each, at daylight regimen of 13 hours, ambient temperature of 20 °C, air humidity 70%, a common feeding front and drinking front of 0.9 m. No vaccinations have been performed.

### *Blood sampling*

Blood samples were obtained at 7-day intervals: day 0 (prior to infection), and days 7, 14, 21 and 28 after the infection.

The total number of blood samples appropriate for testing was 78: 48 from healthy birds (39 control and 9 from birds subject to infection on day 0) and 30 from infected population of birds (obtained on post infection days 7, 14, 21 and 28).

#### *Serological tests*

Three tests were used, one of them (HIT) for detection of subtype-specific antigens and two (IDT and ELISA) – for detection of type-specific antigens.

*HIT.* The reaction was performed on blood sera with 1% avian red blood cells, inactivated for 30 min at 56 °C and 0.05 mL volumes of components (Anonymous, 2005). As antigen, allantoic fluids of virus-infected CE, that after performing the HIT test were further diluted for obtaining 8 haemagglutination units were used. The titre of haemagglutination-inhibiting antibodies was the highest serum dilution, that inhibited the agglutination of 75%–100% of red blood cells. The mean arithmetic titres (MAT) were also calculated as ratio of titres sum to the number of tested sera.

*IDT.* The method of Beard (1970) was used, dissolving 0.6 % Noble (Difco) agar in 7.2 % NaCl and conserving it with 0.001% sodium ethylmercurithiosalicylate (merthiolate).

A live concentrated antigen was obtained from chorioallantoic membranes (CAM) of CE, infected with the viral isolate. The CAM, washed with phosphate buffered solution with pH 7.4, were mechanically homogenized to obtain a dense suspension. To it, an aliquot of phosphate buffered solution with pH 7.4 was added. The antigen was stored at –20 °C without addition of conservants.

Polyclonal polyspecific hyperimmune serum (HIS) was obtained from chickens by the method of Pearson & Senne (1986) with successive inclusion of the strains

A/dusk/Bulgaria/05 H6N2, A/duck/Czechoslovakia/56 H4N6 and A/duck/England/56 H11N6.

Agar (17 mL) was poured in a Petri dish (100×15 mm). Seven wells were cut – one central and 6 peripheral. The diameter of wells was 5.0 mm, and the distance between the central and peripheral wells – 2.4 mm. In the central well, 0.05 mL antigen was put whereas in two opposite peripheral wells – HIS. Patient sera were placed in the other peripheral wells (up to 4). The loaded Petri dish was incubated in a moist camera at 20 °C–25 °C for 72 h.

Apart qualitatively, IDT of positive sera was performed also quantitatively, determining the level of precipitins. The assay included serial dilutions of sera from 1:2 to 1:256 in physiological saline. The MAT were also calculated, accepting the positive undiluted sera as MAT=1.

*ELISA.* A test kit for detection of antibodies against avian influenza was used (Antibody Test Kit, IDEXX, № 09269-EA477). The sera were diluted 1:500. The relative amount of antibodies in tested samples was determined by calculating the ratio of sample (S) to positive control (P). Serum samples with S/P ≤ 0.5 were considered negative and those with S/P > 0.5 – positive.

For measurements and calculations, a xChek 3.3 software and TECAN reader at a wavelength of 650 nm were used.

#### *Statistical analysis of data*

The statistical analysis of data obtained from the different diagnostic tests was done only in chickens, challenged with a viral antigen. The Statistica v.6.0 software was used (StatSoft, Tulsa, OK, USA). The confidence interval (CI) was calculated at a probability of 0.95.

The relative sensitivity, relative specificity and test coverage were calculated

according to Courtney *et al.* (1990) as followed:

- relative sensitivity (S) in % – ratio of the number of positive sera from the infected population  $\times$  100 to the total number of tested sera from the infected population;
- relative specificity (Sp) in % – ratio of the number of negative sera from the non-infected population  $\times$  100 to the total number of tested sera from the non-infected population;
- false negative results (FN) in % – ratio of the number of negative sera from the infected population  $\times$  100 to the total number of tested sera from the infected population;
- false positive results (FP) in % – ratio of the number of positive sera from the non-infected population  $\times$  100 to the total number of tested sera from the non-infected population;
- test agreement ratio % - ratio of the sum of number of sera with equal results from two tests to the total number of tested sera by these two tests.

## RESULTS

The results from the HIT, IDT and ELISA are presented in Table 1. They show that 24 samples from infected chickens were positive in the HIT (S = 80.6 %), 14 were positive in IDT (S = 46.7 %) and 14 – positive in ELISA (S = 46.7 %). False positive results in non-inoculated birds were not present (100% specificity for all tests).

The HIT-positive sera in infected chickens (Table 2) on the 7<sup>th</sup> day were only 4 out of 9 samples (S= 44.4 %) with serum titres from 1:4 to 1:16 and MAT – 1:8. By the 14<sup>th</sup> day, 6 chickens exhibited positive results (S = 66.7 %). The titres were between 1:8 and 1:128. By the 28<sup>th</sup> day, 5 birds were positive (S = 55.6 %). Titres of seropositive chickens ranged between 1:4 and 1:128. On the 14<sup>th</sup> day, MAT was 1:42.7, and on the 28<sup>th</sup> day – 1:44.8. All birds were positive (S=100%) only on the 21<sup>st</sup> day with titres between 1:4 and 1:256. MAT value was the highest – 1:44.89.

Throughout the entire experiment, samples with titres 1:8 and 1:16 predomi-

**Table 1.** Sensitivity and specificity of the haemagglutination inhibition test (HIT), the immunodiffusion test (IDT) and ELISA in 78 sera from chickens – healthy and intravenously infected with 0.1 mL avian influenza isolate A/duck/Bulgaria/05 H6N2, tested at 1-week intervals after the infection for a period of 4 weeks

Test		Healthy population (n=48)	Infected population (n=30)	Relative sensitivity (S); false negative (FN)	Relative specificity (Sp); false positive (FP)
HIT	+	0	24	S: 80.6 %	Sp: 100 %
	-	48	6	FN: 20.0 %	FP: 0 %
ELISA	+	0	14	S: 46.7 %	Sp: 100 %
	-	48	16	FN: 53.3 %	FP: 0 %
IDT	+	0	14	S: 46.7 %	Sp: 100 %
	-	48	16	FN: 53.3 %	FP: 0 %

**Table 2.** Time- and titre-dependent distribution of haemagglutinins (HIT-positive sera) in chickens, intravenously infected with 0.1 mL avian influenza isolate A/duck/Bulgaria/05 H6N2

Antibody titres	Days after the infection				
	0	7	14	21	28
1:4	0 %	50 %	0 %	11.1 %	40 %
1:8	0 %	25 %	33.4 %	22.2 %	0 %
1:16	0 %	25 %	16.7 %	44.4 %	20 %
1:32	0 %	0 %	16.7 %	0 %	0 %
1:64	0 %	0 %	16.6 %	11.1 %	20 %
1:128	0 %	0 %	16.6 %	0 %	20 %
1:256	0 %	0 %	0 %	11.1 %	0 %

**Table 3.** Time- and titre-dependent distribution of precipitins (IDT-positive sera) in chickens (n=9), intravenously infected with 0.1 mL avian influenza isolate A/duck/Bulgaria/05 H6N2

Items	Days after the infection				
	0	7	14	21	28
Total number of seroreagents	0	3 (33.3 %)	5 (55.6 %)	3 (33.3 %)	3 (33.3 %)
Antibody titres					
Undiluted sera	0 %	0 %	20 %	11.1 %	100 %
Diluted sera with titre 1:2	0 %	66.7 %	40 %	66.7 %	0 %
Diluted sera with titre 1:4	0 %	33.3 %	40 %	33.3 %	0 %

nated (29.2 % of each dilution). The values of the individual titres in this trial were from 1:4 to 1:144 at a MAT values for the experiment of 1:38.2.

IDT-detected precipitating titres (IDT-positive sera) in infected chickens (Table 3) in undiluted and diluted up to 1:4 samples by the 7<sup>th</sup> day were 3 (S = 33.3 %), by the 14<sup>th</sup> day – 5 (S = 55.6 % – the highest determined percentage). By the 21<sup>st</sup> and the 28<sup>th</sup> days they were 3 again (S = 33.3%). Four inoculated birds (44.4%) (No No 1, 7, 9, and 11) remained negative whereas three (33.3%) were positive (No No 2, 8, and 10) throughout the entire trial. The others were positive for shorter periods.

By the 7<sup>th</sup> day, the titres of precipitins in positive samples were from 1:2 to 1:4. On the 14<sup>th</sup> day, apart the 1:2 and 1:4 titres, a positive sample was observed in an undiluted serum. On the 21<sup>st</sup> day, positive results in undiluted sera prevailed and the others were in 1:2 titres. At the last testing (day 28), only undiluted sera were found to be positive. The mean titres for the experiment were 1:2.

The number of ELISA-positive chickens (Table 4) on the days of testing were as in the IDT. Two chickens (No No 2 and 10) were positive for the entire period, another 2 (No No 4 and 8) – in two consequent testings. The chickens No No 6 and 11 were positive only in one of all tests. Three chickens (No No 1, 7, and 9)

remained seronegative through the entire experiment.

On the 7<sup>th</sup> day after the infection, positive S/P values ranged between 0.507 and 0.603. On the 14<sup>th</sup> day they were from 0.555 to 1.640, and during this period, the highest values were observed: 40% of tested samples had S/P ratios > 1.000. By the 21<sup>st</sup> day, positive samples had ratios of 0.794–1.239 (33.3 % with S/P ratios > 1.000), and by the 28<sup>th</sup> day they ranged between 0.518 and 1.051 (33.3 % with S/P > 1.000). In birds, positive throughout the entire experimental period (No No 2 and 10) there was a tendency towards lower values on the 7<sup>th</sup> day, increasing on

day 14 (No 2) and 21 (No 10), and decreasing again in subsequent periods.

The comparison of data obtained in the HIT, IDT and ELISA is done in Table 5. The relative specificity of both IDT and ELISA vs HIT was 98.2 % , and the relative sensitivity of these two tests – 54.2 % for each. The coverage between IDT and HIT, and between ELISA and HIT was equal – 84.6 %.

From all tested sera, 14 were positive in both ELISA and IDT vs 24 positive in HIT. The negative results were 64 in both ELISA and IDT and 54 in HIT. There was a difference in 12 sera, 11 of which (91.7%) were positive in HIT and

**Table 4.** Time-dependent distribution of ELISA-positive chickens, intravenously infected with 0.1 mL avian influenza isolate A/duck/Bulgaria/05 H6N2 with regard to the result of the tested samples vs the positive control (S/P)

Chicken	Days after the infection				
	0	7	14	21	28
No 2	S/P = 0.204	S/P = 0.509	S/P = 1.640	S/P = 1.239	S/P = 1.051
No 4	S/P = 0.323	S/P = 0.603	S/P = 1.309	S/P = 0.432	S/P = 0.426
No 6	S/P = 0.107	S/P = 0.389	S/P = 0.915	S/P = 0.406	S/P = 0.410
No 8	S/P = 0.284	S/P = 0.417	S/P = 0.904	S/P = 0.794	S/P = 0.468
No 10	S/P = 0.292	S/P = 0.507	S/P = 0.555	S/P = 0.934	S/P = 0.717
No 11	S/P = 0.228	S/P = 0.441	S/P = 0.458	S/P = 0.459	S/P = 0.518

Legend: The maximum value of S/P in the test with negative result was 0.474.

**Table 5.** Potential of ELISA and IDT compared to HIT for detection of antibodies in chickens, intravenously infected with 0.1 mL avian influenza isolate A/duck/Bulgaria/05 H6N2: relative sensitivity (S); relative specificity (Sp) and between-test agreement

	HIT-negative (n=54)	HIT-positive (n=24)	Relative sensitivity (S); false negative (FN)	Relative specificity (Sp); false positive (FP)	Between-test agreement
ELISA +	1	13	S: 54.2 %	Sp: 98.2 %	84.6 %
ELISA -	53	11	FN: 45.8 %	FP: 1.8 %	
IDT +	1	13	S: 54.2 %	Sp: 98.2 %	84.6 %
IDT -	53	11	FN: 45.8 %	FP: 1.8 %	

**Table 6.** Potential of ELISA and IDT for detection of type-specific antibodies in chickens, intravenously infected with 0.1 mL avian influenza isolate A/duck/Bulgaria/05 H6N2: relative sensitivity (S); relative specificity (Sp) and between-test agreement

	IDT- negative (n=64)	IDT- positive (n=14)	Relative sensi- tivity (S); false negative (FN)	Relative speci- ficity (Sp); false positive (FP)	Test agreement
ELISA +	1	13	S: 92.9 %	Sp: 98.4 %	97.4 %
ELISA -	63	1	FN: 7.2 %	FP: 1.6 %	

negative in both ELISA and IDT. On the contrary, only one serum (8.3 %) was negative in HIT and positive in ELISA and IDT. A part of infected chickens showed negative results in IDT (n=4) and ELISA (n=3) during the entire period of the study, being positive in HIT.

Correlation of results at  $P < 0.05$  at a moderate level ( $r = 0.32787$ ) was observed between HIT and IDT and between HIT and ELISA.

The results of detection of type-specific antibodies by means of ELISA and IDT showed that 13 sera (out of 14 positive ones) matched in both tests. The relative sensitivity of ELISA and IDT was almost equally high – 92.9 % (Table 6). False negative results in both tests were obtained in 7.2 % of sera. The relative specificity was also high and amounted to 98.4 %. The agreement of both tests was 97.4 %. In 2 sera, different results have been obtained: one was negative in ELISA and positive in IDT whereas another one – positive in ELISA and negative in IDT.

The experiment showed that the correlation of data between both tests was high –  $r = 0.7771$  at  $P < 0.001$ .

## DISCUSSION

The chickens infected intravenously with an AIV isolate A/duck/Bulgaria/05 H6N2 underwent an immune transformation and

the resulting raised antibodies (haemagglutinins, precipitins, IgG antibodies) were detected by means of ELISA.

Our data did not confirm the results of Beck & Swayne (1997) about earlier maximum levels only in the IDT (day 5), later results in ELISA (day 10) and the latest – in the HIT. They neither proved the data of Adair *et al.* (1989), Adair *et al.* (1990), Arenas *et al.* (1990) and Meilin *et al.* (2004) for the advantage of ELISA vs HIT or the communication of Lamichhane & Kirkegaard (1997) about the equal potential of both tests. Our data were also different from those of Snayder *et al.* (1985), Adair *et al.* (1990), Boer *et al.* (1990), Beck & Swayne (1997) and Zhou *et al.* (1998) with regard to the higher sensitivity of ELISA vs the IDT.

Our data, similarly to those of Meulemans *et al.* (1987), Zhou *et al.* (1998) and Sala *et al.* (2003) evidenced more positive results in the HIT compared to both ELISA and IDT. One of the possible reasons could be that haemagglutinins are situated on the surface of the viral particle. They are the first to provoke the antigen challenge. The type-specific antigens (located in the core of virions) are hardly available to immunocompetent cells. The contact with them is realized after destruction of the virion.

The intravenously applied viral suspension presumes a rapid immune trans-

formation and maximum results in the immediate period after its application. Nevertheless, in the beginning there was a small number of seroreagents in all three tests. Later, their number increased in our as well as in the experiments of Beck & Swayne (1997). By the 21<sup>st</sup> day of our trials, haemagglutinins were present in all birds and a maximum percentage of positive birds with type-specific antigens (positive in the IDT and ELISA) – 55.6% in each test, were observed on the 14<sup>th</sup> day. Yet, in the trials of Beck & Swayne (1997), the maximum values were observed earlier (by post infection day 10).

Our data about the equal potential of ELISA and IDT correspond to those of Meulemans *et al.* (1987) for the period up to the 11<sup>th</sup> day, because the authors found this statement true only in the early period of the experiment. We presume that the earlier and more prolonged detection of precipitins in our experiment was due to the properties of the obtained antigen.

Not all sera of the infected population contained antibodies and the results about their presence differed among tests (46.7 % for both IDT and ELISA and 80.6 % for HIT). In spontaneous infections, Ziegler *et al.* (1999) have observed various incidence of positive sera (maximum 55 % and minimum 4–5 %). Beck & Swayne (1997) found a difference only in ELISA with 97.7% seropositivity vs 100 % seropositivity in both IDT and HIT.

In our experiments, the precipitins were quantitated for the first time. There were differences in their concentrations with time, the maximum titres being present on the 7<sup>th</sup> day.

Maximum S/P ratio in ELISA was observed by the 14<sup>th</sup> day in this study as well as in that of Beck & Swayne (1997), the difference being only in the values. S/P ratio obtained by Beck & Swayne (1997)

was lower (0.654–1.041) than ours (0.555–1.640).

The highest haemagglutinin titres of 1:256 were observed by the 21<sup>st</sup> day. These results probably influence the mean arithmetic titres of haemagglutinins. They tended to increase up to day 14 (peak values 1:42.78) and to maintain similar values until the end of the study (day 28).

Unlike Meulemans *et al.* (1987) and Brugh (1995) (who determined titres of 1:512–1:4096 with fewer inoculums), the haemagglutinin concentrations in our experiments were 4-fold lower. The similarity with the data of cited authors was only in the tendency of increase with time. We suppose that one of the causes for the observed discrepancy was the kind of used strains and their adaptation to hosts. In other trials (Otsuki *et al.*, 1982; Lu & Castro, 2004) similar to our data have been reported. In cases with high haemagglutinin levels, the isolates were obtained from chickens and the experiment was performed with chickens. This peculiarity is confirmed by the 3–4-fold difference reported by Brugh (1995) in the respective results after intravenous application of various strains (mean titres 1:50 and 1:160). That could further provide a rationale for both our results and those of Otsuki *et al.* (1982), obtained with strains isolated from wild waterfowl.

Comparing our results about the sensitivity, specificity and the agreement of ELISA vs IDT and HIT with those of Zhou *et al.* (1998), it could be seen that in both studies, the results obtained in HIT were with higher values than those in ELISA. At the same time, we found out lower percentages from those of Zhou *et al.* (1998) – by 45.8 % for sensitivity, by 1.6 % for specificity and by 15.3% for test agreement) between ELISA and HIT. In our view, the better performance of HIT



could be attributed to the different types of antibodies that are detected by the test (subtype-specific) compared to ELISA (type-specific) and to the fact that the first contact of the immune system is realized with subtype specific antigens.

Our comparative data about ELISA vs IDT are different from those reported in the literature. We determined equal potentials of these tests with regard to the three parameters, whereas according to Zhou *et al.* (1998), ELISA had a better specificity (98.2 % for IDT vs ELISA) and test agreement (99.9 % for IDT vs ELISA). Our results showed a lowered sensitivity (by 7.1 %) and agreement of data (by 2.5 %) compared to those obtained by Zhou *et al.* (1998), but a higher specificity by 0.2 % (98.4 % in IDT vs 98.2 % in ELISA).

In our opinion, one of the causes for observed variations in sensitivity, specificity and test agreement is the different methods of antigen production. There is still not a standardized procedure for obtaining an antigen for use in IDT. This could be clearly seen from obtained different results (with a difference of 6 %) by Meilin *et al.* (2004) after assaying sera with two types of ELISA obtained in different ways.

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