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Original Contribution

DETECTION OF ANTIBODIES AGAINST AVIAN ISOLATE OF INFLUENZA A VIRUS H6N2 IN TURKEY POULTS AFTER INFECTION VIA DIFFERENT ROUTES

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ABSTRACT

Studies for detection of antibodies against an isolate of influenza A virus H6N2 were carried out by ID, ELISA and HI with 38 experimental turkey poults, 9 of which were infected intravenously, 9 tracheally, 8 orally, 6 by contact and 6 control.

Titre, antibody and S/P values by ELISA differed from test to test and route of infection. ID detected 100% positive poults after intravenous, 85.71% after tracheal, 83.33% after oral infection and 66.67% positive contact poults. Lower values were obtained with ELISA in the orally infected poults (80%) and with HI in the contact poults (50%). The highest per cent HI positive results were obtained on the 14th day and on the 21st day with ID and ELISA.

HI detected more positive sera than ID and ELISA in the intravenously and tracheally infected groups. ID detected more positive contact birds than HI and ELISA. The highest precipitin titres varied in relation to the route of infection starting with the tracheal, oral, intravenous and spontaneous (contact) infection. The S/P of ELISA and the haemagglutinin titre reached high values after intravenous infection and lower values after tracheal, spontaneous and oral infection.

Highest sensibility to infection in turkey poults showed HI followed by ID (93.75% - 72%) and ELISA (89.66% - 68%). In the contact poults ID detected more positive birds than ELISA (88.89%) and HI (77.77%). ELISA and ID results varied from 86.67% - 88.,89%.

Key words: Avian influenza virus, turkey poults, HI, ID, ELISA.

INTRODUCTION

Because of their high sensitivity turkey poults are often used in experiments with isolates of avian influenza A viruses (AIV). With AIV nucleoprotein infection into the host antibodies are formed both against the surface viral antigens (haemagglutinin and neuraminidase) and against the deeper antigens (matrixen and nucleoproteiden) which antibodies can be detected by diagnostic tests, such as the haemagglutination inhibition test (HI), the immunodiffusion (ID) and the enzyme linked immunosorbent assay (ELISA). Experimental results from HI, ID and ELISA vary. The HI test has advantage compared to ELISA (1, 2); sensibility of ELISA equals 99.4%, specificity 100% and agreement 99.6%. In other cases the advantage is on behalf of ELISA (3).

When ELISA is compared to ID, ELISA has the advantage (2, 4). Zhou et al. (2) determine 99.5% sensitivity, 99.4% specificity and 99.5% agreement of ID; later higher results ID - 100% sensitivity, 99.8% specificity and 99.8% agreement are obtained (4).

We compared the diagnostic value of the standard HI, the commercial ELISA and a modified ID with intravenously, tracheally and orally infected with avian influenza isolate A/duck/Bulgaria/05 turkey poults.

MATERIAL AND METHODS

1.Virus. A/duck/Bulgaria/05 isolate (5) of influenza A virus from a wild duck *Anas plathyrynchos* of 10^5 titre and embryo infectious half dose (EID_{50}/0.1ml) was used, calculated by Reed & Muench (6).

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2.Experimental birds. Thirty-eight 30 day-old turkey poults of the Beltsville breed were used; 9 poults were infected intravenously, 9 tracheally, 8 orally with 0.1 ml of allantoic fluid from chicken embryos (CE) infected with the cultivated virus; 6 birds received allantoic fluid from intact CE (control) and other 6 were in contact control. The birds were kept in two 4 x 4 m isolated rooms, at 13-hour daylight, 20 ^{o}C, 70% humidity, 1.8 m long feeding and watering front. Vaccines were not applied.

3. Samples. Blood samples were collected and studied for presence of antibodies on day 0 (prior infection) and after infection on day 7, 14, 21 and 28. From a total of 172 samples, 39 were collected from each of the intravenously and tracheally infected groups, 34 from the orally infected, and 30 from each the contact control and control groups; 62 samples were obtained from the healthy population, 32 from the 0-day group and 30 from the control group; 110 samples were obtained from the infected population, 30 from each the intravenously and tracheally, 26 from the orally infected and 24 from the contact groups.

4. Serological tests. The subtype specific antibodies were detected with HI and the type specific antibodies with ID and ELISA.

4. 1. HI was carried out according to OIE standards (7).

4. 2. ID was carried out as described by Beard (8) with modification (live antigen). Precipitin levels were determined by serial serum dilutions from 1:2 – 1:256 in saline. Average geometric titres (GT) were calculated: GT of positive undiluted sera equals 1.

Antigen and hyperimmune serum was supplied by Animal Health Service (The Netherlands). Noble (DIFCO) 0.6 % agar which was dissolved in 7,2 % NaCl and preserved with 0,001% solution of sodium ethylmercuritiosalicylate – Fluka chemie (ERG) was used. Aliquots of 17 ml of agar were poured in Petri dishes. One central and six peripheral wells 5,0 mm in diameter and at 2,4 mm distance between the central and the peripheral wells were cut and 0,05 ml of antigen were poured into the central well and positive serum with antibody into two opposite peripheral wells. Patient's serum was added to the empty peripheral wells (up to 4). The loaded Petri dishes were incubated in a wet chamber at 20 ^{0}C – 25 ^{0}C for 72 h. ID was used in the qualitative determinations except in the positive sera in which quantity of precipitins was determined by serial dilutions of sera in buffer solution from 1:2 – 1:256.

4. 3. ELISA. We used a test-kit for detection of antibodies against avian influenza (Antibody Test Kit, IDEXX, # 09269-EA477). Sera were diluted 1:500 and the relative antibody content equaled the S/P ratio (sample/positive control). Serum samples of S/P $\leq$ 0.5 were considered negative and those of S/P $\geq$ 0,5 were considered positive. Tests and calculations were carried out with the help of software x Check 3.3 and TECAN reader at 650 nμ wavelength.

4. 4. Statistical analysis. The comparative results from the three diagnostic tests were processed by StatMost program version 2.50®. Comparison of sensibility, specificity and agreement results was carried out by the method of Courtney and Cornell (9).

RESULTS

Antibodies were determined on the 7th day in the infected and on the 14th day in the contact poults by all ID, ELISA and HI. On day 21 maximum ID positive results – 9 (100%) were obtained in the intravenously infected poults, 7 (85.71%) in the tracheally infected, 6 (83.33%) in the orally infected and 4 (66.67%) in the contact poults.

The summarized results showed that HI has higher sensitivity in the intravenously and tracheally infected poults (97.22% and 80.36%) compared to ID (90.97% and 63.09%) and ELISA (87.75% and 60.66%) **(Table 1)**. In the orally infected group and in the contact birds more positive results were obtained by ID. In the orally infected ID detected 60.92% positive poults followed by HI (56.99%) and ELISA (56.76%) and in the contact poults ID detected 37.50% positive, ELISA 33.33% and HI 29.17%.

ID studies on all sera detected 90% positive results in the intravenously, 60.00% in the tracheally, 57.69% in the orally infected group and 37.50% in the contact group. Highest GT was determined in the tracheally (1:23.5) and orally (1:23.33) followed by the intravenously infected (1:13.13) and contact poults (1:8). The reciprocal titres in the tracheally infected reached 1:128 on the 21st day, in the orally infected and intravenously infected 1:64 on the 7th day and in the contact poults 1:8 on the 14th day.

Table 1. *Summarized positive poults results obtained in serological studies by HI, ID and ELISA after infection with avian influenza A virus strain A/duck/Bulgaria/05 via different routes of infection.*

Infected	Test	Means
Tracheally	HI	**80,36***
	ID	**63,09**
	ELISA	**60,66**
Venouslly	HI	**97,22**
	ID	**90,97**
	ELISA	**87,75**
Orally	HI	**56,99**
	ID	**60,92**
	ELISA	**56,76**
Contact	HI	**29,17**
	ID	**37,50**
	ELISA	**33,33**

Legend : * - percent

Highest ELISA S/P values of 3.302 were determined in the intravenously infected on the 21st day, 3.115 in the tracheally infected on the 21st day, 2.791 in the contact poults on the 28th day, and 1.517 in the orally infected on the 28th day.

HI detected highest AAT of 1:736 in the intravenously infected poults on the 7th day with reciprocal titres from 1:128 – 1:2048; in the tracheally infected poults AAT equaled 1:253.71 on the 14th day with reciprocal titres from 1:16 – 1:1024; in the contact poults AAT reached 1:256 on the 28th day with reciprocal titres from 1:64 – 1:256; and lowest AAT of 1:128 in the orally infected poults on the 28th day with reciprocal titres from 1:8 – 1:256.

When ID and ELISA were compared to HI 100% specificity was determined (Table 2). Highest sensitivity was obtained with HI, from 93.75 – 72% with ID and from 89.66% - 68% with ELISA depending on the route of infection.

Table 2. *Summarized positive poults results from comparison of ELISA and ID to HI used for antibody detection after infection with avian influenza A virus strain A/duck/Bulgaria/05 via different routes and contact.*

Test	HI			
	Infected	Sensitivity	Specificity	Agreement
ELISA	Venouslly	**89,66 %***	100 %	95,24 %
	Tracheally	**68,00 %****	100 %	87,30 %
	Orally	**87,50 %*****	100 %	95,45 %
ID	Venouslly	**93,10 %****	100 %	96,84 %
	Tracheally	**72,00 %****	100 %	88,89 %
	Orally	**93,75 %*****	100 %	97,73 %

Legend: * - P< 0,05; ** - P< 0,01; *** - P< 0,001

Comparison between ID and ELISA (both detecting type-specific antibodies) showed higher sensitivity of ID in all experimental birds (Table 3). The specificity and agreement results differed from the sensitivity results.

Highest specificity (maximum) was demonstrated in the contact poults, 97.78% in the tracheally infected and 94.44% in the intravenously infected poults; agreement between 97.92% - 92.06% in the above order.

Table 3. *Summarized positive results from comparison of ELISA to ID sensibility, specificity and agreement results after infection with avian influenza A virus strain A/duck/Bulgaria/05.*

Test	ID			
	Infected	Sensitivity	Specificity	Agreement
ELISA	Venouslly	**88,89 % ****	**94,44 %**	92,06 %
	Tracheally	**88,89 %*****	**97,78 %**	95,24 %
	Orally	**86,67 %*****	**96,55 %**	93,18 %
	Contact	**88,89 %*****	100,00 %	97,92 %

Legend: ** - P< 0,01; *** - P< 0,001

DISCUSSION

The positive results varied in relation to the route of infection, day of appearance and test. Their values tended to increase with time based on precipitin detection by ID, IgG antibodies as detected by ELISA, and haemagglutinins by HI. With HI after the intravenous and tracheal infection maximal number of poults with haemagglutinins and higher antibody titre are detected earlier compared to the tests detecting precipitins and IgG antibodies (ID and ELISA).

It can be suggested that the high values obtained later by the tests detecting type-specific antibodies are due to the fact that their antigens are localized deep in the virion, accessible to deep antigen has only after destruction of the virion (2, 10). After intravenous and tracheal infection the surface viral antigens (haemagglutinins) come directly in contact with the immunocompetent cells from the blood stream, the tracheal mucosa and the lung parenchyma which sooner results in larger number of positive birds and higher antibody titres. In the orally infected and contact poults smaller numbers of positive birds and sera and antibody titres of lower values were obtained by all tests because of the weak antigen stimulating in the orally infected and the delayed access of the virus in the organism of the contact birds.

Higher values were obtained by the tests for detection of type-specific antibodies compared to the test for detection of subtype-specific antibodies in the contact birds (also lower in the orally infected) because of the massive antigen teasing after the virion capside is destroyed. The similarity of results obtained from the orally infected and contact birds suggest that in the spontaneously infected contact poults most of the virus penetrates via the oral route.

Capua et al. (12) in spontaneous cases of infection obtain results similar to ours in the contact group with close reciprocal haemagglutinin values, respectively 1:128/0.05 ml and 1:256/0.05ml.

The HI test proved to be positive until the 14th day after the intravenous and tracheal infection confirmed the results of Capua et al. (12) and the results of Meilin Jin et al. (14) from spontaneously infected birds. On the 7th day we obtained less positive results (88.89%) than Karunukaran et al. (13), who detect 97.99% positive poults, and Mohan et al. (11) - 93.75%.

The ID results also differed: on the 7th day after infection we obtained higher values (88,89%) than Karunukaran et al. (13) and Mohan et al. (11), who obtained 52.35%, but on the 14th day results coincide (100%).

The results from comparing HI to ELISA and ID confirm the results of Zhou et al. (2, 4) of HI advantage and differ in ELISA and ID results. Zhou et al. (2, 4) determine higher sensitivity of ELISA compared to ID sensitivity (100% - 99.4%). We obtained higher ID sensitivity antigen compared to ELISA (88.9% - 86.67%). The differences in the comparative sensitivity, specificity and agreement results are due to the different ways of test preparation. Meilin et al. (14) also found out a 6% difference between the results with two different ELISA kits.

REFERENCES

1. Abraham, A., Sivanandan, V., Karunakaran, D., Halvorson, D. A., Newman, J. A.: Comparative serological evaluation of avian influenza vaccine in turkeys. Avian Diseases, 1988; 32: 659-662.
2. Zhou, E.M., Chan, M., Heckert, R.A., Riva, J., Cantin, M.F.: Evaluation of a competitive ELISA for detection of

antibodies against avian influenza virus nucleoprotein. Avian Diseases, 1998; 42:517-522.
3. Adair, B.M., Todd, D., McKillop, E.R., McNulty, M.S.: Detection of influenza A type-specific antibodies in chicken and turkey sera by enzyme linked immunosorbent assay. Avian Pathology, 1989; 18: 455-463.
4. Zhou, E.M., Chan, M., Heckert, R.A., Riva, J., Cantin, M.F.: A Competitive Enzyme Linked Immunosorbent Assay for Avian Influenza Serological Surveillance. In: Proceeding of the Fourth International Symposium on Avian Influenza, May 28-31, 1997; The University of Gorgia, Athens, GA, USA, American Association of Avian Pathologists, Lib. Cong. Cat. Card No. 97-051243, 1997; 305-312.
5. Zarkov, I., Manvell, R., Shell, W., Bochev, I.: Isolation of avian influenza virus in Bulgaria. The Veterinary Record, 2005; 158: 106-107.
6. Reed, L. J., H. Muench.: A simple methods of estimating fifty percent endpoints. Journal of Hygiene, 1938, 27, 493-497.
7. Anonymous, OIE: AIV, Manual of diagnostic procedures. Version adapted - 05. 2005; Chapter 2.7.12: 1-28.
8. Beard, C.W.: Demonstration of type-specific influenza antibodiey in mammalian and avian sera by immunodiffuson. Bull. Wld. Hlth. Org., 1970; 42: 779-785.
9. Courtney, C.H., Cornell, J.A.: Evaluation of heartworm immunodiagnostic tests. JAVMA, 1990; 197: 724-729.
10. Maulemans, G., Carlier, M.S., Gonze, M., Petit, P.: Comparison of hemagglutination–Inhibition, Agar Gel Precipitin, and Enzyme-linked Immunosorbent Assay for Measuring Antibodies against Influenza viruses in Chickens. Avian Diseases, 1987; 31: 560-563.
11. Mohan, R., Saif, M., Erickson, G., Gustafson, G., Easterday, B.: Serologic and epidemiologic evidence of infection in turkeys with an agent related to the swine influenza virus. Avian Disease, 1981; 25: 11-16.
12. Capua, I., Mutinelli, F., Marangon, S., Alexander, D.A.: H7N1 avian influenza in Italy (1999-2000) in intensively reared chickens and turkeys. Avian pathology, 2000; 29: 537-543.
13. Karunakaran, D., Halvorson, D.A., Sivanandan, V., Newman, J.A.: Pathogenicity of avian influenza viruses isolated from wild mallard ducks and domestic turkey. Avian Disease, 1988; 32: 319-323.
14. Meilin Jin, Wang, G., Zhang, R., Zhao, S., Li, H., Tan, Y., Chen, H.: Development of enzyme-linked immunosorbent assay with nucleoprotein as antigen for detecton of antibodies to avian influenza virus. Avian Diseases, 2004; 48: 870-878.