Comparison of haemagglutination inhibition (HI), immunodiffusion (ID) and ELISA tests for detecting anti avian influenza antibodies in chicken after inoculations with inactivated antigen

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SUMMARY

Intravenous and subcutaneous (2 weeks later) inoculations were carried out in chickens with inactivated antigen from A/duck/England/56-H1N6, for comparing the serological diagnostic values of haemagglutination inhibition (HI), immunodiffusion (ID) and ELISA tests. Sera were collected before IV inoculation and weekly for 5 weeks in 10 chickens and in 2 negative controls. No false positive sample was detected whatever the test used (specificity: 100%) and the overall sensitivity was 92%, 54% and 56% for HI, ID and ELISA respectively. All inoculated birds gave positive results with the HI test the 7th day after IV inoculation with antibody titres ranging from 1:2048 to 1:32, and they remained positive during the whole experiment except for one bird; the subtype specific antibodies tended to increase in 60% of birds 3 weeks after SC inoculation. Whereas ID test detected only 3 samples on the 7th and the 14th days, the test sensitivity increased to 70% since the 1st week after SC inoculation, but 3 birds remained always negative. The ELISA sensitivity was weak during the 2 first weeks after IV inoculation then reached 80% on the 2nd and the 3rd weeks after SC inoculation. Comparison of ID to ELISA gave a high relative sensitivity (89%) and specificity (95.5%) with 93% of agreement, whereas relative sensitivities of ID or ELISA to HI were weaker (59% and 61% respectively) with moderate agreements (75% and 74% respectively). These results show that the biological diagnostic value of the HI test was highest than those of ID and ELISA tests and that the subtype specific antibodies revealed by HI were greatly induced by IV inoculation, while type specific antibodies evidenced by ID and ELISA prevailed after SC inoculation.

Keywords : avian influenza virus - ELISA - immunodiffusion - haemagglutinin inhibition - sensibility - specificity.

RÉSUMÉ

Comparaison de la détection d’anticorps contre le virus grippal aviaire induits par inoculations successives par des antigènes inactivés chez le poulet par les tests d’inhibition de l’hémagglutination, d’immunodiffusion et ELISA. Par I. STOYANOV ZARKOV.

Les antigènes inactivés issus du virus A/duck/England/56 - H1N6 ont été inoculés par voie IV puis par voie SC 2 semaines plus tard, à des poussins afin de comparer les valeurs diagnostiques des tests d’inhibition de l’hémagglutination (IH), d’immunodiffusion (ID) et ELISA. Les sérums ont été recueillis avant l’inoculation en IV puis toutes les semaines pendant 5 semaines chez les oiseaux traités et chez 2 contrôles négatifs. Quelque soit le test utilisé, aucun faux positif n’a été détecté (spécificité : 100%) et les sensibilités globales ont été de 92% pour l’IH, 56% pour l’ELISA et 54% pour l’ID. Tous les oiseaux inoculés ont donné une réponse positive avec le test d’IH le 7ème jour avec des anticorps antihémagglutinateurs allant de 1:2048 à 1:32 et, à l’exception d’un animal, ils sont restés positifs durant toute l’expérimentation : la production des anticorps spécifiques d’un sous-type ainsi détectés a tendu à augmenter 3 semaines après l’inoculation SC chez 60% des oiseaux traités. Alors que l’ID n’a détecté que 3 sérums positifs le 7ème et le 14ème jours, la sensibilité du test a atteint 70% dès la 1ère semaine après l’inoculation SC, mais 3 oiseaux sont toujours restés négatifs. La sensibilité et l’agrément du test ELISA, faible les 2 premières semaines, a atteint 80% 2 et 3 semaines après l’inoculation SC. La comparaison entre les tests ID et ELISA a révélé un fort accord (93%) avec une sensibilité et une spécificité relatives de 89% et de 95.5%, alors que les sensibilités relatives des tests ID et ELISA par rapport à l’IH se sont avérées plus faibles (59 et 61%) de même que les valeurs d’accord (respectivement 75 et 74%). Ces résultats montrent que la valeur diagnostique du test IH est supérieure à celles de l’ID et de l’ELISA et que les anticorps spécifiques d’un sous-type révélés par l’IH sont principalement induits après une inoculation IV tandis que les anticorps spécifiques d’un type détectés par ID et ELISA prévalent après l’inoculation SC.


Introduction

Screening tests for serum antibodies against avian influenza A viruses (AIV), are performed to prove infection [7]; when disease is recreated [13]; to determine the species of sensitive birds [1, 9, 18] and to detect the immune response under different conditions such as species of inoculated birds [9, 18, 21], age of birds, dose of inoculums [12] and beginning of antibody production [13, 14]; and to indicate efficiency of vaccine production [2, 11, 17, 19].

Serological methods are usually used for detection of type-specific antibodies produced against nucleoprotein antigen (NP) and subtype antibodies against haemagglutinin (H) and neuraminidase (N). The immunodiffusion (ID) [4, 6, 14] and ELISA [1- 4, 14, 15, 20, 21] tests used type-specific antigen, while the haemagglutinin inhibition (HI) [7, 14 - 16] and the neuraminidase inhibition - subtype-specific antigen.

ID is preferred for its simple and fast realization and for the possibility of studying large numbers of samples. However, the method needs large quantities of antigen and serum and results are not obtained before the 24th h. Alternatives to ID are the indirect and the competitive modifications of the ELISA method using purified viral antigen [21]. Recently the possibility of applying the above methods to ostriches, emus and other species subjected to large-scale production is studied. Comparative studies on chicken and
turkey sera by ID and ELISA show differences in sensibility and specificity [1, 4, 14, 20, 21]. ELISA sensibility is in the range of 98.2% - 99.5%, its specificity is 99.4% with 99.1% - 99.5% of agreement. The specificity decreases to 97.7% in ostriches and to 85.5% in emus with 86.1% and 97.7% of agreement, respectively.

Comparison of sensibility and specificity between ELISA and HI varies from equal values [3, 14] to higher [2] or lower [20] for ELISA. Others have developed and used an ELISA test and HI for detection of monoclonal antibodies against 77 and shown the advantages of HI with 99% of agreement. By ELISA antibodies are not determined in samples of titre from 1:16 - 1:32.

Efficiencies of HI, ID and ELISA are compared in an attempt to determine the diagnostic value of each test in the detection of anti avian influenza virus antibodies in chickens after intravenous (I.V.) and subcutaneous (S.C.) inoculations. The I.V. inoculation is more important for antibody production against subtype-specific antigens (HI) and the S.C. inoculation - against type-specific antigens (ELISA, ID).

Material and methods

CHICKEN AND EXPERIMENTAL DESIGN

The experiment was carried out with 12 ten-day old chicken of the Dekalp breed kept in a separate room (4 m²) on a deep litter during September and October at 20 °C, 70% humidity and 13 hours of daylight. Ten chickens were inoculated with the inactivated viral antigen and two were contact controls. The first inoculation was carried out intravenously (I.V.) and subcutaneously (S.C.) inoculations. The I.V. inoculation is more important for antibody production against subtype-specific antigens (HI) and the S.C. inoculation - against type-specific antigens (ELISA, ID).

ANTIGEN

Virulent strain A/duck/England/56 - H11N6 with EID50 - 10.7.75 and haemagglutination reaction (HA) of 1:512 titre was used. The virus was inactivated in 0.2% from 10% buffered formalin in PBS at pH 7.2 (0.02% final concentration) for 3 days at 37 °C at the same HA titre. Inactivation was checked by infecting six 9-day old chicken embryos with 0.1 ml inactivated viral suspension. Embryos were observed for 7 days (144 h) and they were remaining alive. Positive haemagglutination was not found out in the allantoid fluid.

SEROLOGICAL TESTS

Three kinds of tests were applied: ELISA and ID for detection of NP type-specific antibodies and HI for subtype-specific antibodies.

ELISA

We used a test-kit for detection of antibodies against avian influenza (Antibody Test Kit, IDEXX, Flock Check, US Vet License N°313, Product Code: 5004.00). Sera were diluted 1:500 and the relative antibody content in the unknown sample (S) was determined by calculating the S/P ratio where P was a positive control. Serum samples of S/P ≤ 0.5 were considered negative and those of S/P ≥ 0.5 were considered positive. Tests and calculations were carried out by software x Check 3.3 and TECAN reader at 650 nm wavelength.

Immunodiffusion (ID)

Antigen and positive poly-specific hyper-immune serum for avian influenza was supplied by Animal Health Service (The Netherlands). The modification of BEARD’s method [8] was used with 0.6 % Noble (DIFCO) which was dissolved in 7.2 % NaCl and preserved with 0.001% solution of sodium ethylmercuritosalicylate - Fluka chemie (ERG). Aliquots of 17 ml of agar were poured in petri (100x15 mm). 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 were incubated in a wet chamber at 20 °C - 25 °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 - 1:32.

Haemagglutination inhibition (HI)

This reaction was carried out with serum inactivated at 56 °C for 30 min in quantities of 0.05 ml with 4 hemagglutinating units from A/duck/England/56-H11N6. Sera dilutions started from 1:4 with 1% avian erythrocytes [5].

STATISTICAL ANALYSIS

Statistical significance of methods was evaluated by StatMost program version 2.50. Comparison of sensitivity, specificity and agreement of methods was carried out by the method of COURTNEY et al. [10] with 50 samples obtained from birds inoculated with the viral antigen from the 7th to the 35th day.

Results

As early as the 7th day all inoculated birds were positive by HI (100% Se). Serum titres varied from bird to bird in the range of 1:2048 to 1:32, and they were above 1:64 in 50% of birds (median value: 1:96). Later, on the 2nd week, one bird only became negative (90% Se). Thereafter, the subtype specific antibody titres tended to increase in 60 and 70% birds on the 14th and the 21st days respectively (ranges: 1:512 - 1:16). Two and 3 weeks after SC inoculation, (days 28 and 35 after IV inoculation), they rose again in 30 and 60% of birds respectively (ranges: 1:512 - 1:32 at Day 28 and 1:1024 - 1:32 at Day 35) (figure 1). The average arithmetic titre (AAT) also varied from bird to bird (1:38.4 - 1:813.2) at 1:257.74 for the whole experiment.
The precipitin titres by ID varied from undiluted sera to 1:16 diluted sera (Figure 2). ID detected three positive samples (30% Se) on the 7th and 14th day after I.V. inoculation while 7 days after the S.C. inoculation (on the 21st day of the whole experiment) their number increased to 7 (70% Se). Three inoculated birds remained always negative. During the first days, sera of lower titre prevailed, while after the S.C. inoculation maximal titres were recorded. On the 7th day precipitin titres ranged from undiluted to 1:4, then on the 14th day all positive sera were undiluted, on the 21st day (7 days after the S.C. inoculation) and on the 35th day - from undiluted to 1:16 and only on the 28th day - from 1:2 to 1:16.

With ELISA, 4 samples were positive on the 7th day. On the 21st day their number increased to 6 and remained as high as 8 from the 28th day till end of experiment. Two inoculated birds remained always negative. The sensibility consequently varied from 20 % (day 14) to 80 % (day 28 and 35) (Figure 3).

Overall results from inoculation of chicken with inactivated antigen are given in Table I. Sensibility (frequency of positive sera obtained from the inoculated birds) (Se) and specificity (frequency of negative sera from non-inoculated birds) (Sp) of HI, ID and ELISA were determined in 22 samples from non-inoculated birds (10 before inoculation and 2 non-inoculated) and 50 samples from 10 inoculated birds. Se and Sp were calculated as well as the number of false negative results (negative tests in inoculated birds) and false positive results (positive tests in non-inoculated birds). The results from the three tests showed that 46 samples (Se 92%) contain haemagglutinins, 28 were positive with ELISA (Se 56%) and 27 with ID (Se 54%), whereas the specificity was always excellent (100%) for all tests (no false positive results was recorded in the healthy population). The highest positive correlation was obtained between ELISA and ID results ($r = 0.93$, $p < 0.001$) while moderate correlations were evidenced between HI and ELISA ($r = 0.54$, $p < 0.05$) and between HI and ID ($r = 0.47$, $p < 0.05$).

Comparison between HI and ELISA or ID was performed. Obviously, for a relative specificity (frequency of ELISA or ID negative sera from HI negative samples) of 100%, the relative sensibilities (frequency of ELISA or ID positive sera from HI positive samples) were moderate (61 and 59% respectively) and the respective agreements (number of identical score) were 75% (with ELISA) and 74% (with ID) (Table II). The comparison of the detection of type-specific antibodies by ID and ELISA show that the relative sensibility of ID compared to ELISA was high (89%) and the relative specificity slightly diminished (95.5%) with a strong agreement of 93% (Table III).

**Discussion**

Highest percent of positive sera was obtained with HI (92%) in comparison to ELISA (56 %) and ID (54 %) in contrast to the results of ADAIR *et al.* [3, 4] and MAULE-MANS *et al.* [14]. Some sera remained negative with ELISA and ID even at high HI titres in contrast to the results of SALA *et al.* [15]. Differences could be explained with the intravenous route of first inoculation and the first contact with the surface viral H and N antigens. In this way, on the 7th day, all the birds were HI positive, while 60% and 70%
were negative with ELISA and ID. Type-specific antibodies appeared on the 21st and on the 28th day, (7th-14th day after the S.C. inoculation) when maximum number of positive samples was obtained by HI. BECK et al. [8] published similar high positive values with HI and ID after intravenous inoculation with non inactivated antigen. In our case the formalin inactivation might have reduced the destruction of the surface viral antigens and the subsequent release of the type-specific antibodies.

Another important fact was the fast reduction of HI titre on the 14th-21st day after the I.V. inoculation followed by an increase on the 28th day (14th day after the S.C. inoculation). Similar tendency was observed with the other methods. ID showed few positive sera and lowest precipitin levels on the 7th and on the 14th day; and one week after the S.C. inoculation (on the 21st day) maximum level was determined. The number of ELISA positive birds increased from 20% on the 14th day (minimum) to 60% on the 21st day and 80% (maximum) on the 28th day.

Comparison between results from HI to ELISA or ID showed a low relative sensitivity (61 and 59% respectively), a high specificity (100%) and a moderate agreement (75 and 74% respectively). These results might be explained by the fact that we compared methods able to detect different types of antibodies and we applied a new method of processing and inoculation of the antigen. BECK et al. [8] also do not find out significant correlation between HI and ELISA.

Results from ELISA and ID for detection of antibodies of similar types agreed. The relative sensitivity and specificity were 89% and 95.5% respectively with an agreement of 93%. We confirmed in this way the results of MEULEMANS et al. [14], ABRAHAM et al. [1] ZHOU et al. [20] who evidenced a high or similar ELISA sensibility with not inactivated antigens. By contrast, LAMICHHANE et al. [12] reported a lower sensibility when inactivated vaccine antigen was used.

**Conclusions**

1. When an intravenous and a successive subcutaneous inoculation with inactivated AIV antigen were carried out, the HI test allowed the detection of all inoculated birds on
the 7th day. But, when all samples from inoculated birds were considered, the sensitivity was reduced to 92%. For ID and ELISA tests, the sensitivities observed were 54 and 56% respectively. Nevertheless, for all the 3 tests used, the specificity was 100% indicating the lack of false positive results.

2. HI antibody count was high after intravenous inoculation, decreased in the next weeks, and rose again 2 to 4 weeks after subcutaneous inoculation.

3. Type-specific antibodies determined by ELISA and ID prevailed after subcutaneous inoculation.

4. Comparison between results from HI, ELISA and ID show a strong agreement between ELISA and ID, and significant and moderate agreements between ELISA and HI and between ID and HI.

References


