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DETECTION OF ANTIBODIES TO AVIAN REVERSE TRANSCRIPTASE BY INDIRECT ELISA

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Summary. — An improved indirect ELISA test for detection of antibodies to the reverse transcriptase (revertase) is described. The sensitivity of the revertase ELISA test was compared to that of the revertase inhibition test. Serum samples of various origin and sera of specific pathogen free (SPF) hens were examined for the revertase antibodies. The presence of these antibodies in the sera of SPF chickens is discussed.

Key words: ELISA test; reverse transcriptase; SPF chicken

Introduction

The presence of reverse transcriptase and/or of corresponding antibodies indicates that retrovirus replication or at least a partial expression of the provirus has occurred (Graevskaya and Sito, 1977). Antibodies to avian revertase of the myeloblastic virus were detected in the sera of experimentally infected chickens and in the sera of commercial broilers (Graevskaya and Sito, 1977). In serum samples of SPF chickens, free of exogenic retroviruses and their antibodies, Graevskaya *et al.* (1982) detected antibodies to avian revertase by means of the reverse transcriptase inhibition test.

In this paper we describe an indirect ELISA-test for detecting revertase antibodies. The principle and technique of the method were described in detail by several authors (Engvall and Perlmann, 1971; Weeman and Schuurs, 1971; Voller *et al.*, 1976; Mohr and Zschiesche, 1981; Tam, 1984).

Materials and Methods

Sera. The investigations were carried out with sera of different origin: SPF hens previously investigated by the revertase inhibition test, the offspring of the latter, laying hens from various egg production farms and broilers.

Virus multiplication and purification. Preparation of the reverse transcriptase. The avian myeoblastosis virus (AMV), strain BAI was used as described by Heider *et al.* (1978). The plasma obtained containing 3 to 4.5×10^{11} virus particles was stored at −80 °C. For purification and concentration of the virus by ultracentrifugation the method of Chuc (1982) was applied. The isolation of the enzyme was described by Graevskaya *et al.* (1975).

Preparation of immune sera. The immunoglobulin (Ig) fraction was isolated from chicken and/or rabbit serum according to Krögner and Knöpke (1972). The anti-chicken or anti-rabbit Ig was obtained from goats according to Chuc (1982). The antiserum against the revertase was raised in chickens. The chickens were intravenously immunized with 440 units of revertase at 10-day intervals. The animals were boostered with the half dose diluted 1 : 1 with Freund's adjuvant. The first blood sample was collected 10 days later and two further samples were collected at one-week intervals. Anti-revertase rabbit sera were prepared as described by Graevskaya *et al.* (1975). Anti-AMV rabbit serum was obtained after injection at weeks intervals 600 μg AMV into the rabbit footpad. Three weeks after last injection blood was drawn at three--days intervals, 5 ml each.

Preparation of the conjugate. Highly purified horse radish peroxidase (Serva, 1000 units/mg) was used. For conjugation of the goat anti-rabbit or anti-chicken Ig the periodide one-step method (Schmeer, 1982; Herrmann, 1982) was applied. The conjugate was used in dilution of 1 : 1000.

ELISA. The indirect ELISA method of Engvall and Perlmann (1972) was used for detection of revertase antibodies. Plastic plates with 20 wells of 0.6 ml volume were coated with reverse transcriptase antigen. The enzyme solution was adjusted to pH 9.6 and protein concentration of 5 μg/ml with carbonate-bicarbonate buffer. Rabbit sera were tested after immunization with AMV, rabbit and chicken sera after immunization with the revertase. Chicken sera free of revertase antibodies as detected in the revertase inhibition test served as negative controls. Dilution of 1 : 20 up to 1 : 2560 in phosphate buffered saline (PBS) Tween-20 containing 10% horse serum were prepared. The test was carried out with goat anti-chicken or goat anti-rabbit Ig peroxidase conjugate in 100 μl volume, using moist incubation chambers. Phosphate citrate buffer was used to prepare the substrate and for dilution of the conjugate. For washing PBS Tween-20 containing horse serum was employed. The substrate solution contained 0.04% orthophenylendiamine (OPD) and 0.15% H_2O_2; 0.5 mol/l H_2SO_4 was used to stop the reaction. The photometry measurements were performed on Spekol (Carl Zeiss Jena) at 480 nm wavelength.

Results

The first investigation were carried out to optimize the ELISA technique. To establish the specificity of the revertase reaction, we started with the already approved „rabbit system". The anti-revertase rabbit serum with a known antibody titre was compared with negative rabbit serum. As second control served the anti-AMV rabbit serum. As follows from Fig. 1, high antibody titre in both sera were clearly demonstrated also by the ELISA test. The extinction value of the negative control sera was substantially lower, the values remaining constant only after 1 : 160 dilution.

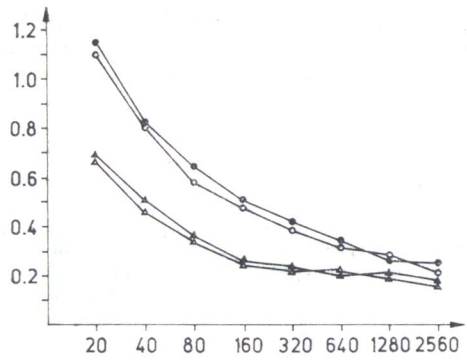

Fig. 1.
Indirect ELISA tests with anti-AMV serum and anti-revertase rabbit serum (RS)
● AMV-RS (positive); ○ RT-RS (positive); (▲) RS (negative); (△) pooled negative RS.
Abscissa: serum dilutions; ordinate: E_{480} nm.

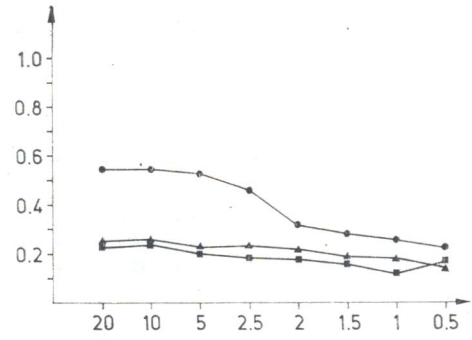

Fig. 2.
Results of ELISA at decreassing revertase concentrations
● RHS — $F_{10/11}$; ▲ negative SPF-HS; ■ negative Prague-BS (pool)
Abscissa: enzyme (revertase) dilution (μg/ml); ordinate: E_{480} nm.

Specificity of the antigen-antibody reaction with the indirect ELISA test was demonstrated by control rabbit serum. To determine the optimum antigen concentration, the wells were covered with different quantities of revertase and evaluated with a pooled positive serum and negative sera.

Fig. 2 shows the extinction values with a series of decreasing revertase concentrations. For the chosen 1 : 160 serum dilution, the negative control remained constantly low. The values of the positive pooled sera decreased only at 5 μg/ml antigen concentration. The optimum revertase concentration in our test was between 5 and 10 μg/ml. A complete conformity of the results was obtained when the both revertase concentrations were compared (Fig. 3). Therefore, the low dose of 5 μg/ml was used in the latter tests. These experiments also showed that the serum should be diluted 1 : 160, taking into consideration the intensive non-specific background colour. Using the values of different negative sera, the extinction limit could be determined as the threshold value between positive and negative results.

The extinction values decreasing to 1 : 160 serum dilution remained thereafter at an almost constant level; at this dilution the negative sera had their extinction limit between 0.18 and 0.26. Results of the examination of positive and negative sera are compared in Fig. 4. For the established limit

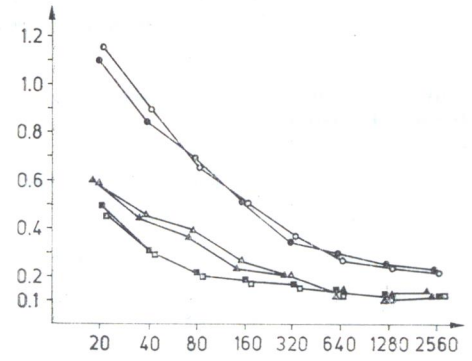

Fig. 3.
Extinction value of different antigen concentrations and increasing serum dilutions (10 and 5 μg enzyme/ml).
● ○ positive RHS; ▲ △ negative SPF-HS; ■ □ negative Prague-BS (pool).
Abscissa: serum dilution; ordinate: E_{480} nm.

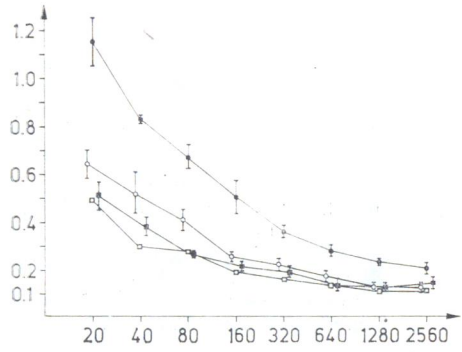

Fig. 4.
Comparison of positive and negative chicken sera in the ELISA test
● RHS — $F_{10/11}$ or $F_{3/5}$; ○ negative SPF-HS; ■ negative KWH-BS; □ negative Prague-BS (pool).
Abscissa: serum dilution; ordinate: E_{480} nm.

values all negative sera were found below the limit and all positive sera at least by 50 % above.

Further investigations dealt with serum samples of various origin to be tested for antibodies to revertase. First we examined sera positive in the reverse transcriptase inhibition test, in which no *gs* antigen or ALV antibodies could be detected by classical methods (neutralization, immunofluorescence, COFAL tests). The sera of these animals were examined on the one hand in the reverse transcriptase inhibition test and, on the other hand, in the reverse transcriptase ELISA test. Out of 16 sera examined, 5 were positive in the former and 6 in the latter test, indicating that the sensitivity and the specificity of both tests was well comparable.

In an ELISA mother-daughter comparison, the female offspring of 8 mothers were tested in the ELISA and the revertase inhibition tests. The serum samples of both generations showed complete agreement in detecting positive or negative transvertase antibodies.

In sera of SPF hens, free of exogenous ALV or of antibodies to ALV, antibodies to the avian revertase were detected with ELISA in 14 % of the samples. We also investigated random samples of sera from poultry enterprises. As shown in Table 1, from 0 to 10 % of the field samples were positive. The positive reaction depended on the origin of poultry tested and corresponded to the natural presence of ALV infection in the flocks. An

Table 1. Results of investigations of poultry serum samples for the presence of revertase antibodies by ELISA-test

Serum	Age of animal	Positive	Total	Per cent
broiler	8 weeks	—	13	—
laying hens		4	19	20
parents	35 —	3	10	30
parents	48 —	4	10	40

observed increase of positive titres with growing age corresponded to the leucosis findings in the flock.

Discussion

The aim of this investigation was to examine the practical use of indirect ELISA for detection of antibodies to revertase and optimize the method for this purpose. A comparison of the revertase inhibition test and the revertase ELISA confirmed that the sensitivity of the enzyme immunoassay corresponded to that of inhibition test operating on a molecular basis. ELISA was well adapted to detect antibodies against the revertase. Alike to each antibody-antigen system. optimalization and standardization of ELISA was necessary. The determination of the endpoint between positive and negative serum titre was indispensable. In our case, an extinction of 0.3 for serum dilution of 1 : 160 represented the positive titre. The results of the present revertase antibody test with field poultry sera were in accord with the former results of the revertase inhibition test (Graevskaya *et al.*, 1982). In juvenile sera the antibody titres were substantially lower than in sera of older animals. Unlike these authors we were unable to detect antibodies in sera of pullets with ELISA-test, probably due to lower sensitivity of the immunological as compared to molecular test.

The RNA genome of all retroviruses contains 3 genes participating in the replication process; the *gag* section for the synthesis of the nuclear protein, the *pol* section for the synthesis of the reverse transcriptase and the *env* site for the synthesis of the envelope protein. It is known (Baluda, 1973) that in normal chicken cells these genes are not always expressed. Their expression may be blocked at several loci; from production of the *gs* antigen only (*gag* section) up to complete virus particle formation. It is difficult to explain the presence of revertase antibodies in the serum of SPF chickens free of leukosis viruses and their antibodies. If partial expression of the *pol* gene exists in chickens and the revertase is synthesized in the absence of virus particles, antibodies against revertase may be induced upon the release of the enzyme from the host cell. In such case no antibodies against the virus will be found. Partial expression of the *gag* gene has been described on several cocasions and demonstrated by radioimmunoassay (Varmus, 1982; Coffin, *et al.*, 1983). As the animals tested have been free of exogenous leukosis virus and corresponding antibodies already for several generations, it seems probable that we were dealing with partial expression of the *pol* gene.

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