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# SIMPLIFICATION OF IMMUNE ADHERENCE HEMAGGLUTINATION TEST FOR DETECTION OF RABIES ANTIBODIES IN HUMAN SERUM

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## SUMMARY

In the present work the immune adherence hemagglutination test (IAHA) was standardized in a simplified procedure. This test showed good reproducibility, better than the classical mice serum neutralization test (SN). The tests showed high correlation degree: high titers in one test corresponded to high titers in the other one, and the same occured with low titers. The IAHA test is extremely simple, fast to perform, and of low cost when compared to tests such as SN or indirect immunofluorescense (IIF). It also proved to be useful in less sophisticated laboratories or even as a screening test for the titration of rabies antibodies.

**KEY WORDS:** Immune adherence; Hemagglutination test; Rabies antibodies.

## INTRODUCTION

The immune adherence phenomenon was first described by LAVERAN & MESNIL[9] and LEVADITI[11]. Later PEREIRA[12] and ITO & TAGAYA[7] carried out the first studies in order to apply this test in virological research.

Basically the test consists in activating the complement system through the formation of antigen-antibody complexes resulting in production of the C3b component which promotes the agglutination of human erythrocytes. It should be pointed out that only primate erythrocytes show receptors on their surface for the C3b fraction.

Several studies showed that the reaction of hemagglutination by immune adherence is more specific and sensitive than complement fixation (CF) test, as well as much simpler to perform[3, 6, 10, 12].

Recently LENNETTE & LENNETTE[10] standardized the immune adherence hemagglutination (IAHA) test for several bacterial and viral antigen-antibody systems.

In 1983 BUDZKO et al.[2] adapted this test for rabies serology, suggesting its use for routine detection and quantification of rabies antibodies.

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The objective of the present study was to adapt a simplified procedure for IAHA test to detect and titrate antirabies antibodies in human sera, comparing it with the serum neutralization (SN) test, wich is the most commonly used among us[1].

## MATERIALS AND METHODS

1 — Sera: Thirty samples of human sera were analyzed in order to estimate the reproducibility of IAHA simplified method as compared to the original one. Another 35 samples were evaluated in duplicates (double-blind) in order to compare the IAHA by the simplified method with the SN. The results were quantitative and qualitative analyzed.

2 — Antigens: Antigen and control antigen was obtained from Pfizer Quimica Ltda — São Paulo, Brasil. Briefly, fixed rabies virus cultured in bovine kidney cells were inactived by $\beta$-propiolactone and purified as previously described[5]. Titers were determined by block titrations prior to use.

3 — Complement: Fresh guinea-pig serum was used as source of complement. The complement was stored at −70°C in small portions after was reconstituted.

4 — Diluents: Kolmer saline[8], Veronal buffer saline (VBS), Gelatin-Veronal buffer (GVB), Bovine serum albumin-Veronal buffer (A-VBS), Dithiothreitol-ethilenediaminetetraacetic acid-VBS (D-VBS)[2].

5 — Erythrocytes: Twenty human blood samples were collected in equal volume of sterile Alsever solution from known type "O" donnors and than stored at 4°C for up 5 weeks. These samples were tested, since the test is known to vary with erythrocytes from different donnors. A human serum known to be positive for rabies antibodies was reagent up to 1:80 or 1:160 dilutions in the presence of 14 of the 20 erythrocytes samples tested. With four out of the remaining 6 erythrocytes samples, serum showed lower titers and was non reagent with two of them. Immediately prior to use, the erythrocytes were washed twice in Kolmer saline and than resuspended to a concentration of 1% in Kolmer saline.

6 — Original technique: The IAHA method was performed as described by LENNETTE & LENNETTE[10].

7 — Modified technique: The simplified technique was based on the original technique with few modifications. Briefly, equal volumes (0.025 ml) of serial dilutions of serum and rabies antigen (1:15) were incubated in 96-well plastic plates (Petecil) at 37°C for 30 minutes. After adding 0.025 ml of a 1:120 dilution of fresh guinea pig serum, the mixture was further incubated at 37°C for 40 minutes. A human type "O" erythrocyte suspension (0.025 ml) was then added and the plates were allowed to stand at room temperature for 2 hours. The hemagglutination pattern was read, and the titers were recorded as the reciprocal of the highest serum dilution showing positive agglutination.

Each test was always performed with proper controls of serum, antigen, complement and erythrocytes.

Results were considered as positives when the patterns of hemagglutination obtained were 3+ or 4+ (plus) and negative or absent in controls.

8 — Serum neutralization test: This test was performed essentially as described by ATANASIU[1], with sera diluted up to 1:625. All tests were performed against 25-40 $LD_{50}$ and titer values $< 5$ were considered negative.

## RESULTS

Comparison between the Veronal buffer and the Kolmer solution: Table 1 shows a comparison between the IAHA procedure performed with Veronal buffer with 0.1% BSA (Bovine Serum Albumin), Kolmer solution with 0.1% and 0.01% BSA and Kolmer solution without BSA.

Total agreement was observed among the titers of the 30 sera tested with all diluents except when the serum albumin was completely removed from the Kolmer solution, when the occurence of nonspecific agglutination was observed.

TABLE 1
Evaluation of four different diluents in the Immune Adherence Hemagglutination Test (IAHA) for antirabies antibodies titration in vaccinated and non-vaccinated persons.

| | DILUENT | | | |
|---|---|---|---|---|
| | V 0.1% | K 0.1% | K 0.01% | K − |
| VACCINATED | 20 | 20 | 20 | 80 |
| | 40 | 40 | 40 | 160 |
| | 80 | 80 | 80 | 160 |
| | 160 | 160 | 160 | 640 |
| | 40 | 40 | 40 | 80 |
| | 160 | 160 | 160 | 640 |
| | 640 | 640 | 640 | 640 |
| | 40 | 40 | 40 | 80 |
| | 20 | 20 | 20 | 80 |
| | 20 | 20 | 20 | 80 |
| | 80 | 80 | 80 | 160 |
| | 80 | 80 | 80 | 320 |
| | 80 | 80 | 80 | 320 |
| | 80 | 80 | 80 | 320 |
| | 320 | 320 | 320 | 640 |
| | 160 | 160 | 160 | 640 |
| | 40 | 40 | 40 | 80 |
| | 40 | 40 | 40 | 80 |
| | 20 | 20 | 20 | 80 |
| | 80 | 80 | 80 | 160 |
| | 160 | 160 | 160 | 640 |
| | 80 | 80 | 80 | 320 |
| NON VACCINATED | < 5 | < 5 | < 5 | 20 |
| | < 5 | < 5 | < 5 | 40 |
| | < 5 | < 5 | < 5 | 20 |
| | < 5 | < 5 | < 5 | 40 |
| | < 5 | < 5 | < 5 | 40 |
| | < 5 | < 5 | < 5 | 40 |
| | < 5 | < 5 | < 5 | 80 |
| | < 5 | < 5 | < 5 | 40 |

V = Veronal buffer + percentage of BSA
K = Kolmer saline + percentage of BSA
K − = Kolmer saline without BSA

TABLE 2
Comparison of Immune Adherence Hemagglutination Test (IAHA) and Serum Neutralization Test (SN) in antirabies antibodies titration in vaccinated and non-vaccinated persons by double-blind assay of serum duplicates.

| | IAHA | | SN | |
|---|---|---|---|---|
| | Samples | | Samples | |
| | 1st aliquot | 2nd aliquot | 1st aliquot | 2nd aliquot |
| VACCINATED | 40 | 40 | 67 | 25 |
| | 40 | 40 | 32 | 625 |
| | 40 | 80 | 158 | 125 |
| | 40 | 40 | 10 | 19 |
| | 80 | 80 | 32 | 25 |
| | 80 | 40 | 158 | 83 |
| | 80 | 40 | 275 | 151 |
| | 40 | 40 | 51 | 54 |
| | 20 | 20 | 57 | 25 |
| | 40 | 40 | 275 | 95 |
| | 40 | 40 | 54 | 323 |
| | 20 | 20 | 46 | 166 |
| | 40 | 40 | 12 | 13 |
| | 80 | 80 | 223 | 625 |
| | < 5 | < 5 | 57 | 5 |
| | 160 | 160 | 331 | 625 |
| | 160 | 80 | 625 | 625 |
| | 320 | 640 | 625 | 467 |
| | 160 | 160 | 331 | 20 |
| | 320 | 320 | 625 | 625 |
| | 320 | 640 | 625 | 625 |
| NON VACCINATED | < 5 | < 5 | < 5 | < 5 |
| | < 5 | < 5 | < 5 | < 5 |
| | < 5 | < 5 | < 5 | < 5 |
| | < 5 | < 5 | < 5 | < 5 |
| | < 5 | < 5 | < 5 | < 5 |
| | < 5 | < 5 | < 5 | < 5 |
| | < 5 | < 5 | < 5 | < 5 |
| | < 5 | < 5 | < 5 | < 5 |
| | < 5 | < 5 | < 5 | < 5 |
| | < 5 | < 5 | < 5 | < 5 |
| | < 5 | < 5 | < 5 | < 5 |
| | < 5 | < 5 | < 5 | < 5 |
| | < 5 | < 5 | < 5 | < 5 |
| | < 5 | < 5 | < 5 | < 5 |

For qualitative analysis of the results the YULE association coeficient was calculated and its value was 1 (one). The agreement of the antibody titers of tested sera with Veronal buffer plus 0.1% BSA, Kolmer solution plus 0.1% and 0.01% BSA was 100% (30/30).

Comparison between IAHA and SN reproducibility: Thirty five human sera were analyzed in duplicates (double-blind) and the qualitative analysis of the results were performed.

On the basis of these results the Yule association coeficient was calculated for the IAHA and the SN tests, both values being equal to 1 (one). The agreement were calculated from the values of the antibody titers of sera in duplicates. Thus, for the IAHA test there is an agreement of 70% (14/20), whereas the other 30% showed a discrepancy of only one dilution. For SN there was an agreement of 47% (10/21), a discrepancy of 1 dilution interval in 43% (9/21) and of 2 dilution intervals in 10% of the cases (2/21).

Correlation between IAHA and SN: Still based on table 2 data, the Spearman correlation coeficient was calculated, with the antibody titers of the first duplicate of both tests. This coeficient was 0.76 and its significance was estimated by Student test (5.23) that was significant at the

reject level adopted ( = 0.05), for 21 degrees of freedon (t = 2.080).

## DISCUSSION

The simplified IAHA test here presented proved to be highly satisfactory as an alternative method for rabies antibody titration. Compared to the classical SN test the IAHA test showed much greater reproducibility. Similar results in duplicated tests were observed in 70% and 47% of serum samples tested by IAHA and SN tests respectively. The remaining 30% of the samples tested by IAHA procedure showed differences in the titers corresponding to only one double dilution. In the SN test 43% of the samples showed discrepancy of 1 interval of five fold dilution, 10% of them showed discrepancy of 2 intervals.

The Spearman correlation coeficient indicated that the antibody titers mesured simultaneously by the SN and IAHA tests showed high correlation, i.e., high titers in SN test correspond to high titers in IAHA test and conversely, low titers in SN test correspond to low titers in IAHA test. It can be also noted that this test is much more easily performed, the result being obtained in an average period of 4 hours, while the SN test required the observation of the animals for 2 or 3 weeks.

One out of the sera studied in duplicates presented negative result by IAHA while positive result was obtained by SN even though with low titer (Table 2). Positively of this serum was confirmed by indirect immunofluorescence procedure[4].

In its original description[3] the IAHA test is performed with Veronal buffer. In the present study the Veronal buffer was replaced by the Kolmer solution, which is basically a saline solution with the addition of magnesium. The stages of plate coating with gelatin and of interruption of the reaction with EDTA-Dithiothreitol were eliminated too. These modifications greatly reduced the costs of performing the test, with no impairment to results.

In can be noted in Table 2 that the 10 fold reduction in BSA concentration in the diluent (from 0.1% to 0.01%) does not alter the results either, reducing even further the cost of the test. Total elimination of the BSA was not possible though, since it caused false hemagglutination patterns.

It has been stated by LENNETTE & LENNETTE[10] that only erythrocytes from one donnor out of three give suitable results in the IAHA test. In our work 14 out of 20 erythrocytes tested were satisfactory. Lower titers were obtained with the remainder ones. This difference found in the frequence of usable erythrocytes in the IAHA test in our work and in the LENNETTE & LENNETTE one, might be due to differences in the receptors concentration for C3b in the erythrocytes of the two studied populations.

We believe that the results obtained should recommend the inclusion of this test for routine use the detection and titration of rabies antibodies.

## RESUMO

**Simplificação da Reação de Hemaglutinação por Imunoaderência para detecção de anticorpos anti-rábicos em soros humanos.**

A Reação de Hemaglutinação por Imunoaderência foi padronizada de maneira simplificada, para detecção de anticorpos rábicos em soros humanos. Esta reação mostrou grande reprodutibilidade, maior que a apresentada pela clássica prova de soroneutralização em camundongos (SN).

Ambas as provas mostraram alto grau de correlação, ou seja, altos títulos em uma reação correspondeu a altos títulos na outra; o mesmo ocorreu em relação aos títulos baixos.

A reação de Hemaglutinação por Imunoaderência, além de ser de execução extremamente simples e rápida, tem custo bem menor se comparada a provas como a soroneutralização ou a imunofluorescência indireta. Pode, portanto, ser de grande utilidade em laboratórios com poucos recursos ou mesmo ser utilizada como uma prova de triagem para a titulação de anticorpos rábicos.

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REFERENCES

1. ATANASIU, P. — Titrage des anticorps rabiques pratiqué sur les sérums humains. **Bull. Off. int. Epiz., 67**(34): 383 387, 1967.

2. BUDZKO, D. B.; CHARAMELA, L. J.; JELINEK, D. & ANDERSON, G. R. — Rapid test for detection of rabies antibodies in human serum. **J. clin. Microbiol., 17**: 481 484, 1983.

3. GERSHON, A.; KALTER, Z. G. & STEINBERG, S. — Detection of antibody to varicella-zoster virus by immune adherence hemagglutination. **Proc. Soc. exp. Biol. (N.Y.), 151**: 762-765, 1976.

4. GOLDWASSER, R. A. & KISSLING, R. E. — Fluorescent antibody staining of street and fixed rabies virus antigens. **Proc. Soc. exp. Biol. (N.Y.), 98**: 219 223, 1958.

5. GUIDOLIN, R.; BALTAZAR, M. C. & ZELANTE, F. — Produção da vacina anti rábica veterinária em suspensão de células BHK. **Rev. Microbiol. (S. Paulo), 14**: 27 35, 1983.

6. INOUYE, S.; MATSUNO, S.; HASEGAWA, A.; MYAMURA, K.; KONO, R. & ROSEN, L — Serotyping of dengue viruses by immune adherence hemagglutination test. **Amer. J. trop. Med. Hyg., 29**: 1389-1393, 1980.

7. ITO, M. & TAGAYA, I. — Immune adherence hemagglutination test as a new sensitive method for titration of animal viruses antigens and antibodies. **Jap. J. med. Sci. Biol., 19**: 109 126, 1966.

8. KOLMER, J. A. — The tecnique of the Kolmer complement fixation test for syphilis employing one fifth amount of reagents. **Amer. J. clin. Path., 12**: 109 112, 1942.

9. LAVERAN, A. & MESNIL, F. — Recherches morphologiques et expérimentales sur le trypanosome des rats. **Ann. Inst. Pasteur, 15**: 673-714, 1901.

10. LENNETTE, E. T. & LENNETTE, D. A. — Immune adherence hemagglutination alternative to complement fixation serology. **J. clin. Microbiol., 7**: 282 285, 1978.

11. LEVADITI, C. — Sur l'état de la cytase dans le plasma des animaux normaux et des organismes vaccinés contre le vibrion cholérique. **Ann. Inst. Pasteur, 15**: 894-927, 1901.

12. PEREIRA, O. A. C. — The use of immune adherence reaction in the study of some Brazilian arboviruses. **Rev. Inst. Med. trop. S. Paulo, 8**: 41-52, 1966.

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