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Estimation Of Rabies Antibodies In Animal Sera Using Different Techniques

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ABSTRACT

Rabies antibodies were detected in dog, cat and cattle serum samples and quantified through application of four different methods including Serum neutralization test (SNT), rapid fluorescence focus inhibition test (RFFIT), indirect ELISA and Dot ELISA. The comparison of rabies antibody titers determined in tested samples using RFFIT showed lower than indirect ELISA and SNT. The dot ELISA may have potential applications as a rapid , simple and economical field test in the detection of rabies antibodies but it revealed lower sensitivity and specificity than indirect ELISA and SNT but more than RFFIT. Otherwise, RFFIT needs more time and expensive test. So, the previous study confirmed that indirect ELISA is the best of choice for detection of rabies antibodies in sera of different animal species because of its highly sensitive, specific and safe laboratory technique.

INTRODUCTION

Rabies occurs worldwide and more than 3 billion people live in areas in which the disease is enzootic. Every year about 55,000 people die from rabies, with more than 50% in Asia *(1, 2)*. Historically, prophylaxis against important infectious diseases of dogs, cats and others species has been based on annual vaccination. Farm animals and mainly grazing cattle and sheep were exposed to rabid animals including foxes and stray dogs. This approach has resulted in disease control for infections that causes morbidity and mortality. Studies have shown that, a high percentage of dogs and cats are protected for a number of years following a single vaccination. Veterinarians and researchers have come to the conclusion that, the puppy has adequately responded to vaccination or to confirm the immune status in a mature dog and cat to check the antibody levels. The most commonly used technique for detection of protective level of rabies antibodies in sera of animals and humans is the rapid fluorescent focus inhibition test (RFFIT) developed by *(3)*. The RFFIT was selected as the pharmacodynamic marker assay. This assay is regarded as the standard rabies virus neutralization assay in diagnostic laboratories, vaccine and biotherapeutic characterization, and rabies-related clinical studies *(4)*. The fluorescent antibody virus neutralization (FAVN) test has been shown to be more specific than RFFIT *(5)* but The RFFIT is highly sensitive and advantageous because of its low time required. The ELISA is the most frequently used binding assay available, with numerous published protocols and professionally marketed ELISA kits available to detect rabies antibodies. The specificity of the ELISA is dependent upon the of the target antigen used in the test whole virus or purified viral proteins. Antibodies detected in an ELISA do not necessarily have a neutralizing function. Published reports indicate that cross-reactivity, potentially leading to false positives, may increase in ELISA assays that employ whole virus rather than purified G as the target antigen.

The present study compares between four serological tests (Serum neutralization test (SNT), (RFFIT), Dot ELISA and indirect ELISA), aiming to determine the most rapid sensitive and accurate one for determination and titration of rabies antibodies in animal sera.

MATERIAL AND METHODS

Rabies virus

BHK-21 cell culture adapted Evelyn Rokitincki Abelesth (ERA) strain of rabies virus of a titer 10^7 $TCID_{50}$/ml was supplied by the Department of Pet Animal Vaccine Research; Veterinary Serum Vaccine Research Institute. (VSVRI), Abbasia, Cairo and used in application of SNT and RFFIT.

Rabies antigen

Rabies antigen was prepared from infected BHK-21 cells by ERA strain according to *(6)*. This antigen was used in the dot-ELISA and RFFIT to determine rabies antibodies in sera of vaccinated animals.

Serum samples

Two hundred dogs (150 vaccinated by inactivated rabies virus vaccine and 50 non-vaccinated dogs) and hundred cats (70 vaccinated by inactivated rabies virus vaccine and 30 non-vaccinated) serum samples were obtained through the government veterinary hospital. Abbasia, Cairo. Moreover, hundred fifty serum samples (100 vaccinated by inactivated rabies virus vaccine and 50 non-vaccinated) were obtained from emergency vaccinated cattle in different private farms.

Reference anti-rabies serum

Reference anti-rabies serum was supplied by Copenhagen, Denmark, as 30 IU / ampoule. It was used as positive control in the applied serological tests.

Baby hamster kidney cell culture (BHK)

BHK-21 cell culture was used for detection of anti- rabies antibody by RFFIT and SNT.

Dot ELISA Procedure

The dot ELISA test and optimization studies were based on the method of *(7 ,8)*.

Indirect Enzyme Linked-Immune sorbent Assay (ELISA)

SERELISA® Rabies Antibody Mono Kit was supplied by SYNBIOTICS EUROPE SAS. 2, rue Alexander Fleming F- 69367 Lyon, Cedex 07 allows a quantitative detection of rabies antibodies in individual dog , cat and cattle serum samples. A minimum of 0.5 IU/ml rabies antibodies is required to protect against rabies infection, according to the World Health Organization recommendations *(9)*.

Serum neutralization test (SNT)

It was carried out, using the micro titer technique for estimation of rabies antibodies in the obtained serum samples. Serum samples were diluted through two fold dilutions. The antibody titer was calculated as the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 $TCID_{50}$ of rabies virus *(10)*.

Rapid fluorescence focus inhibition test (RFFIT)

The test was carried out using the method *(3)*, modified by *(11)*. The rabies virus neutralizing antibody titers are mathematically calculated using the Reed and Munch method *(12)* for assigning a RFFIT titer *(10)*.

Calculation of sensitivity and specificity

Sensitivity was calculated with the formula [TP/ (TP+FN)] × 100 where TP was the number of samples with true-positive results as determined by the reference assay and FN was the number of samples with false-negative results. Specificity was defined as [TN/ (TN+FP)] × 100 where TN was the number of samples with true-negative results and FP was the number of samples with false positive results according to *(13)*.

RESULTS AND DISCUSSION

Table 1. Comparison between different serological tests for detection of rabies antibodies in dogs

Serological tests	Sera from non-vaccinated dogs (n = 50)					Sera from vaccinated dogs (n = 150)				
	True Negative (TN)		False Positive (FP)			False Negative (FN)		True Positive (TP)		
	No.	Specificity (%)	No.	(%)	Mean titer: ≥ 0.5IU/ml	No.	(%)	No.	Sensitivity (%)	Mean titer: ≥ 0.5IU/ml
Dot-ELISA	43	86	7	14	±Ve	8	5.3	142	94.6	+++Ve
Indirect ELISA	48	96	2	4	0.6	1	0.6	149	99.3	2.02
SNT	46	92	4	8	0.5	5	3.3	145	96.6	1.9
RFFIT	39	78	11	22	0.5	10	6.6	140	93.3	1.0

Sensitivity = [TP/(TP+FN)] × 100. Specificity = [TN/(TN+FP)] × 100.

- Positive reaction of dot ELISA was visualized (naked eye) as brown dots (doubtful: ±ve ; strong positive +++ve) *(7,8)*.

-The protective rabies neutralizing antibody titer ≥ 0.5 IU/ml (SNT). Protective level of rabies antibodies should not be less than 0.5 IU/ml (RFFIT). *(14)*. IU value = sample serum titer/ Reference serum titer

Table 2. Comparison between different serological tests for detection of rabies antibodies in cats

Serological tests	Sera from non-vaccinated cats (n = 30)					Sera from vaccinated cats (n = 70)				
	True Negative (TN)		False Positive (FP)			False Negative (FN)		True Positive (FP)		
	No.	Specificity (%)	No.	(%)	Mean titer: ≥ 0.5IU/ml	No.	(%)	No.	Sensitivity (%)	Mean titer: ≥ 0.5IU/ml
Dot-ELISA	25	83.3	5	16.6	±Ve	5	7.1	65	92.8	+++Ve
Indirect ELISA	27	90	3	10	0.5	0	0	70	100	1.5
SNT	22	73.3	8	26.6	0.6	3	4.2	67	95.7	1.0
RFFIT	26	83.3	4	13.3	0.5	10	14.2	60	85.7	0.9

Table 3. Comparison between different serological tests for detection of rabies antibodies in cattle

Serological tests	Sera from non-vaccinated cattle (n = 50)					Sera from non-vaccinated cattle (n = 100)				
	True Negative (TN)		False Positive (FP)			False Negative (FN)		True Positive (TP)		
	No.	Specificity (%)	No.	(%)	Mean titer: ≥ 0.5IU/ml	No.	(%)	No.	Sensitivity (%)	Mean titer: ≥ 0.5IU/ml
Dot-ELISA	45	90	5	10	±Ve	20	20	80	80	+++Ve
Indirect ELISA	48	96	2	4	0.6	0	0	100	100	1.2
SNT	46	92	4	8	0.6	7	7	93	93	1.0
RFFIT	43	86	7	14	0.5	11	11	89	89	0.9

Table 4. Conclusive comparison between different serological tests for different animal species.

Serological test	Animal species					
	Dogs		Cats		Cattle	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Indirect ELISA	99.3	96	100	90	100	96
SNT	96.6	92	95.7	73.3	93	92
Dot ELISA	94.6	86	92.8	83.3	80	90
RFFIT	93.3	78	85.7	83.3	89	86

Comparing the serological methods used in diagnosis of infectious diseases, it is important to evaluate particularly their sensitivity, specificity, and accuracy to prevent errors in results obtained by various methodical procedures, it is inevitable to use fully characterized reference standards, reagents *(11)*. According to WHO recommendations, the vaccinated animals are protected sufficiently when their level of rabies antibodies equals to or exceeds 0.5 IU/ml *(9)*. Four different methods, Dot-ELISA, indirect ELISA, RFFIT and SNT were used in our study to detect rabies virus antibodies in dog, cat and cattle sera. The obtained results of these tests showed good correlation between indirect ELISA and SNT *(15)*.

The RFFIT method has been recommended by the U.S. Centers for Disease Control and Prevention (CDC) and the WHO *(16,17)* as the standard assay to measure antibody levels against rabies virus in order to determine whether animals at risk for rabies exposure need a booster vaccination. Despite the availability of alternatives, this method remains the standard method for measuring rabies-specific antibodies *(18)*. But this method demands hours of tedious microscopic observation and is also not suitable for field large scale; surveys and cannot be quantities accurately antibody titers in vaccinated cattle

(19). However, this test was not as sensitive as the indirect ELISA and SNT for assessing the immune status of vaccinated animals. The comparison of rabies antibody titers determined in vaccinated dogs as shown in Table (1), indicate that indirect ELISA presents a relative sensitivity and specificity of 99.3% , 96% respectively. On the other hand, the SNT presents sensitivity and specificity of 96.6%. , 92% respectively for dogs sera. Due to higher specificity and sensitivity of SNT than dot ELISA and RFFIT to measure and evaluate vaccine efficiency but the disadvantages of SNT that it only be performed with live virus and are not recommended for use outside endemic areas or in laboratories without appropriate bio-security facilities and also take more time *(20)*. These obtained results were showed that a strong correlation-ship between the indirect ELISA and SNT but indirect ELISA was found to be more sensitive, specific , accurate and rapid than SNT in agreement with *(15,20)*. Advantages of indirect ELISA was simple, rapid and relatively inexpensive test for the detection of rabies antibodies initiated this work. The great advantage is that the antigen is inactivated and can be used safely in the routine laboratory. On the other side, Dot ELISA presents a relative sensitivity and specificity of 94.6% , 86% respectively. While, RFFIT presents a relative sensitivity and specificity of 93.3% , 78% respectively. These obtained results showed a strong correlation ship between the Dot ELISA and RFFIT but the Dot ELISA was found to be more sensitive, specific and accurate than RFFIT in agreement with *(21,22)*. But, we found that dot-ELISA as a simple, rapid than SNT and RFFIT *(23)*. Due to the RFFIT is time demanding, expensive and unpractical for routine use in virological laboratories *(24)*, a disadvantage of the RFFIT is the reading of results and their evaluation. In the presented work, we compared the standard diagnostic methods (SNT and RFFIT) revealing that there were differences in rapidity of the tests, simplicity an easiness of preparation for them, costs of the reagents and equipment of laboratories. With regard to the preparation and performance of test (RFFIT takes 24–48 hours) and the respective costs. The RFFIT requires lower volumes of examined serum (0.1 cm3). Comparison of mean rabies antibody titers of vaccinated dogs showed indirect ELISA was 2.02 IU/ml higher than SNT (1.9 IU/ml) and RFFIT (1.0 IU/ml). Table (2), shows the obtained results were indicated that indirect ELISA is more sensitive, specific and accurate as the follow indirect ELISA presents a relative sensitivity and specificity of 100% , 90% respectively. SNT presents sensitivity and specificity of 95.7%. 73.3% respectively, for cat sera. There were differences in rapidity of the tests, simplicity and easiness of preparation for them, costs of the reagents and equipment of laboratories. Dot-ELISA test for rabies antibodies detection in cat sera was found to be more specific, sensitive and faster than RFFIT *(25,26)*. Table (3) revealed that indirect ELISA presents a relative sensitivity and specificity of 100% , 96% respectively. SNT presents sensitivity and specificity of 93%. 92% respectively, for cattle sera. These obtained results here, nearly correlated with the results in dogs and cats. Table (4) concluded the sensitivity and specificity of the different serological tests used in this study in different animal species. In conclusively, The previous obtained results were confirmed that indirect ELISA was found to be more sensitive , specific and accurate than others serological tests in different species.

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الملخص العربى

استبيان أجسام السعار المناعية في أمصال الحيوانات باستخدام طرق مختلفة

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أجريت هذه الدراسة على مجموعة من أمصال كلاب وقطط وأبقار محصنة بلقاح السعار المثبط وغير محصنة للكشف عن أجسام السعار المناعية باستخدام كاشف الإنزيم المرتبط المناعي اللطعى (Dot ELISA) و اختبار المصل المتعادل(SNT) و اختبار الفلورسينتى السريع المثبط المحدد (RFFIT) واختبار الاليزا الغير مباشر(Indirect ELISA) لتحديد حساسية و سرعة ودقة كل اختبار تم تجميع مائتين عينة من أمصال كلاب بالمستشفى البيطري منها مائة وخمسون عينة من كلاب محصنة وخمسون عينة أخرى من كلاب غير محصنة ،علاوة على مائة عينة من أمصال قطط بالمستشفى البيطري مقسمه إلى سبعون عينة من قطط محصنة وثلاثون عينة أخرى من قطط غير محصنة، تم تجميع مائة وخمسون عينة من أمصال أبقار بمزارع خاصة منها مائة عينة من أبقار محصنة و خمسون عينة من أبقار غير محصنة. تم استبيان المستويات المناعية المتكونة فى أمصال الحيوانات المحصنة ، اختبار الاليزا قد أعطى اعلي حساسية ودقة وذو خصوصية عالية، اختبار المصل المتعادل ذو ارتباط متقارب مع اختبار الاليزا،وان الإنزيم المرتبط المناعي اللطعى و اختبار الفلورسينتى السريع المثبط المحدد أعطى نتائج الى حدا ما فاعلة وذات ارتباط قوى إلا أن كاشف الإنزيم المرتبط المناعي اللطعى أعطى نتائج أسرع من كل الاختبارات الأخرى لكن ذو حساسية ودقة اقل بالإضافة إلى أن اختبار الفلورسينتى السريع المثبط المحدد يحتاج إلى وقت ومكلف ،كما انه غير عملي ويحتاج لإعادة تقييم النتائج مما ينتج عنه بعض الأخطاء. يوصى باستخدام اختبار الاليزا كاختبار ذو حساسية وخصوصية ودقة ولا يستخدم فيه فيروس حي بالإضافة إلى انه سريع النتائج يليه اختبار المصل المتعادل.