MONITORING OF RABIES IMMUNE STATUS IN EXPERIMENTALLY VACCINATED PUPPIES

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ABSTRACT

Rabies is a lethal viral disease that poses a great risk to both humans and animals. Therefore, there is an urgent need to manage it in the primary hosts, particularly dogs, by immunising them with powerful vaccines and boosting their immune response to provide them a high level of protective immunity. Following up the immune status of experimentally vaccinated different puppy's groups with the locally manufactured inactivated rabies vaccine via the use of serum neutralization test (SNT) revealed that BCG induced earlier and higher levels of antibodies with a men value 106.6 by the second month post vaccination when inoculated simultaneously with rabies vaccine than in case of rabies vaccination alone (53.3). Also, indirect enzyme linked immune sorbent assay (ELISA) showed higher titer (6log10) in puppies receiving BCG vaccine than in those received rabies vaccine alone (4.5log10) by the 2nd month post vaccination. The results of ELISA kit test confirmed that BCG with rabies vaccine induced higher protection percentage (90%) than that induced by rabies vaccine alone (85%) by the first week and second month post vaccination. In addition, latex agglutination test (LAT) was able to determine rabies antibodies in the sera of vaccinated puppies showing strong agglutinations with high antibody titers and weak agglutinations with low antibody titers through the first and last week's post vaccination in a parallel manner to the results of SNT; indirect ELISA and ELISA kit. On the other side, the indirect fluorescent antibody technique (FAT) confirmed that inoculation of BCG simultaneously with rabies vaccine resulted in earlier and higher levels of rabies antibodies than those induced by rabies vaccine alone with positive reactions up to dilutions of 6log10 and 5log10 respectively by the second month post vaccination. So, it could be concluded that BCG can be used as immune enhancing agent to improve the immunological response of vaccinated puppies to rabies vaccine. In addition, all of the used serological tests are able to determine rabies antibodies in the sera of vaccinated animals quantitatively.

Keywords: Rabies virus; serum neutralization, Latex agglutination, Indirect fluorescent antibody technique.

1. Introduction:

Warm-blooded animals can contract the acute lyssavirus illness known as rabies, which can also infect humans (a condition known as zooanthroponosis). It primarily affects the central nervous system (CNS), and almost often, it progresses fatally. In at least 150 countries, the rabies virus (RABV) is present, and rabies claims the lives of more than 55,000 individuals each year. Dogs have been reported to be the most significant vector and are to blame for 95% of rabies-related deaths in people. In fact, rabies kills at least 59,000 people per year, with 36.4% of them occurring in Africa alone **(WHO, 2013).**

The suggested method for avoiding and controlling rabies is to vaccinate pets annually because rabies is thought to be a disease that is preventable by vaccination **(WHO, 2016)**.

It is advised to stay away from keeping wild animals as pets and to avoid handling any dogs, cats, or cattle that may be carriers of the disease as well as any bats that are lying on the ground or acting strangely. Each nation's competent authorities are required to have a mechanism in place for actively monitoring the disease and implementing effective vaccination campaigns in areas where there is a high risk of presentation **(OIE, 2008).**

It was discovered that a rabies vaccine made in inactivated cell culture that used BHK-21 as the cell host and binary ethylene amine as the inactivator was both safe and immunogenic for dogs and cats (**Edries, 1994).**

For cattle, sheep, and horses, the protective dose of a locally produced inactivated cell culture rabies vaccine was established. It was demonstrated that this vaccine could produce significant levels of antibodies that remained for a year after immunisation in vaccinated animals (**Khodier, 1999 and Edries *et al.*, 2001**). In more recent times, a novel Carbopol adjuvanted inactivated cell culture rabies vaccine has been developed, giving inoculated dogs significant concentrations of specific, serum-neutralizing antibodies against rabies after a single dosage (1 ml) **(Naglaa *et al.*, 2020).**

It has been found that a sufficient serum titer of antibodies obtained through active or passive immunisation is associated with resistance to infection and constrained viral multiplication. In order to eventually eradicate the rabies virus, effective cell-mediated immunity is crucial. The single most effective method of preventing rabies in humans worldwide has been parental dog immunisation, it was claimed (**Greene and Rupprecht, 2006)**

In order to determine vaccine effectiveness in dogs, this study aims to compare the immunological responses of puppies who received the rabies vaccine alone and those who received it in combination with the BCG vaccine. A second goal was to create a database on which rabies vaccinations might one day be licenced with longer periods of immunity.

2. Material and Method:

2.1. Animals:

The serum neutralisation test revealed that nine native breed puppies aged three months were free of rabies antibodies. . They were divided into 3 groups (3puppies/group) where the first group was vaccinated with rabies alone receiving 1ml injected s/c in the inner side of the thigh and the second group was vaccinated with rabies and BCG vaccine inoculated intradermally in the right side of the neck using 0.2ml of the reconstituted vaccine containing 8.4 × 106 CFU (**Osman *et al.*, 1987).**

The third group was kept without any treatment as test control.

Sample collection:

Blood samples were collected from all puppy groups before and after immunization at one-week intervals for four weeks, then every month for six months through jugular vein puncture under aseptic conditions, allowed to clot at 4 0C overnight, then centrifuged at 2000 rpm for 15 minutes. The produced specimen were stored at -20 0C until serological tests (ELISA, SNT, IFAT, and RIDT) were performed to monitor the levels of induced rabies immunity in vaccinated puppies.

2.2. Rabies vaccine:

The Department of Pet Animal Vaccine Research (DPAVR); Veterinary Serum and Vaccine Research Institute (VSVRI) provided local inactivated cell culture rabies vaccine (ERA strain) for experimental vaccination of puppies.

2.3. BCG vaccine:

VSVRI provided the Bacillus Calmette-Guerin freeze-dried BCG vaccine (5mg) and its diluents 1-3.

2.4. Rabies virus:

The serum neutralisation test was used to estimate rabies antibodies using a BHK-21 cell culture modified Evelyn Rokintniki Abelseth (ERA) strain of rabies virus with a titer of 107 TCID50 /ml obtained from DPAVR **(Edries, 1994).**

2.5. Cell culture:

VSVRI provided a baby hamster kidney cell line (BHK13), which was utilized to detect rabies antibodies in the sera of vaccinated puppies using a serum neutralization test and the indirect fluorescent antibody technique (IFAT).

2.6. Rabies virus antigen:

Binary Ethyleneimine [BEI] in 0.1M was used to inactivate the produced viral fluid. Binary Ethyleneimine stock solution with 3% viral concentration at 37oC for 1 hour with constant stirring, followed by immediate addition of sterile solution of sodium thiosulphate at final concentration 2% to terminate the action of BEI on the virus. The rabies antigen utilized in the indirect ELISA to detect rabies antibodies in sera samples from vaccinated puppies.

2.7. Enzyme linked immunosorbent assay (Solid phase ELISA):

Anti-dog immunoglobulin [IgG entire molecule] combined with the peroxidase Horse Radish Peroxidase enzyme HRP was used to monitor rabies antibodies in vaccinated puppies according to **(Mani & Madhusudana, 2013).**

2.8. Rabies virus antibody quantitate ELISA kit:

It was supplied by Shezhen Zhenrui Biotech Co. Ltd Add: 7 Floor, Gaoke Budling, Yueliangwan Road, Nanshan District, Shenzhen, China.

2.9. Serum neutralization test (SNT):

SNT in BHK-21 cell culture was done in flat bottom tissue culture micro titer plates (96 wells) utilising the micro technique method as described by (**Ferreira, 1976)** for assessing rabies antibody titers in vaccinated puppies. The antibody titer was calculated as the reciprocal of the final serum dilution required to neutralise and inhibit the rabies virus CPE at 100TCDI50/ml according to **Singh *et al.* (1967)**.

2.10. Indirect fluorescent antibody test (IFAT):

The indirect fluorescent antibody test (IFAT) detects anti-rabies virus (RABV) immunoglobulin G (IgG) and M (IgM) antibodies in cerebral spinal fluid (CSF) and serum specimen in a semi-quantitative, sensitive, and quick manner. An indirect fluorescent antibody test (IFA) was done according to **OIE (2017)**.

2.11. Latex agglutination test:

Latex beads Polystyrene is an aqueous solution with a solids concentration of 10% supplied by Sigma Chemical Company. It was carried out according to (**Sastry & Bhat, 2016).**

3. Result:

Monitoring of antibodies of rabies were found in the serum of vaccinated puppies by serum neutralization test revealed that rabies antibodies in simultaneously vaccinated puppies with rabies and BCG vaccines began earlier and had higher levels of antibodies (with mean value 5.3 and 2, 6 by the first week post and a men value 106.6 and 53.3 respectively by the second month after vaccination) as shown in table (1) and figure (1).

Table (1): Rabies serum neutralizing antibody titers in experimentally vaccinated puppies

Puppy groups	Mean Rabies serum neutralizing antibody titer*						
	0 time	1WPV**	2WPV	3WPV	4WPV	2MPV***	3MPV
Group-1	0	4	8	16	32	64	64
	0	4	8	16	32	64	64
	0	2≤	4	8	16	32	32
Mean	0	2.6	6.6	13.3	26.6	53.3	53.3
Group-2	0	4	8	16	32	64	64
	0	8	16	32	64	128	128
	0	4	16	32	64	128	128
Mean	0	5.3	10.6	26.6	53.3	106.6	106.6
Group-3	0	← 0 →					
	0						
	0						
Mean	0						

-Group-1= vaccinated with rabies vaccine alone. -Group-2= vaccinated with rabies vaccine and BCG. -Group-3 = non vaccinated control.

*SNT titer = the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100$TCID_{50}$ of rabies virus (The recommended. protective SNT titer is at least 16)

**WPV= week post vaccination ***MPV= month post vaccination.

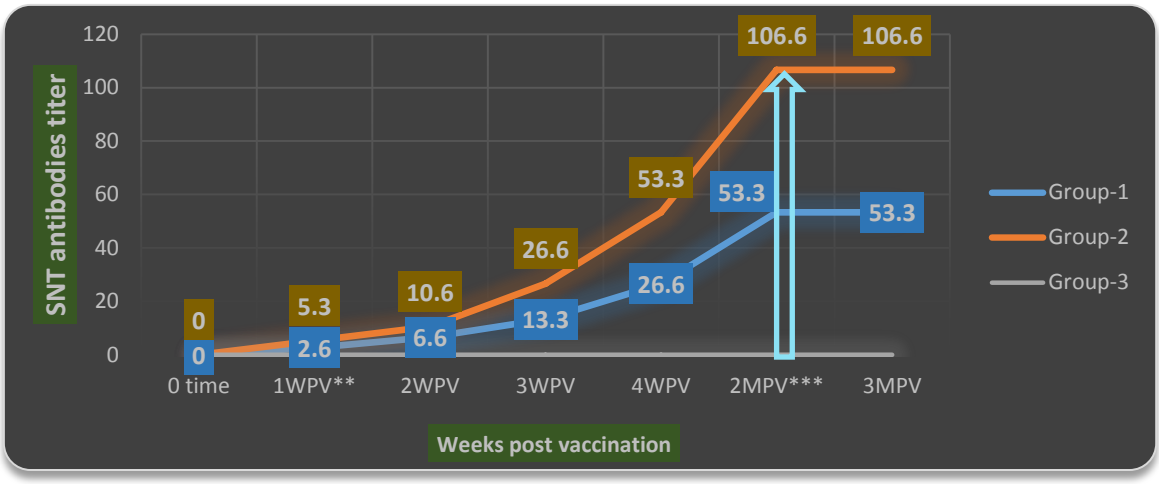

Fig (1): Rabies serum neutralizing antibody levels in vaccinated puppies
-Group-1= vaccinated with rabies vaccine alone.
-Group-2= vaccinated with rabies vaccine and BCG.
-Group-3 = non vaccinated control.

Antibodies of When administered with rabies vaccine, BCG elicited greater levels of rabies antibody (1, 6 and 5.6log10 by the first week and second month post immunization, respectively). than in case of puppy's vaccination with Rabies vaccine alone showing the values of 1 and 4.5 log10 by the first week and second month post vaccination respectively as tabulated in table (2) and figure (2).

Table (2): Rabies ELISA antibody titers in experimentally vaccinated puppies

Puppy groups	Rabies ELISA antibody titer (log10/ml)						
	0 time	1WPV*	2WPV	3WPV	4WPV	2MPV**	3MPV
Group-1	0	1	2	2.5	3.5	4.0	4.5
	0	1	2	2.5	3.0	4.5	4.5
	0	1	2	3	3.0	5	5
Mean	0	1	2	2.6	3.1	4.5	4.8
Group-2	0	2	3	4	4.5	5.0	5.0
	0	2	3	4.5	5.5	6.0	6.0
	0	1.5	2.5	3.5	4.5	6.0	6.0
Mean	0	1.6	2.8	4.0	4.8	5.6	5.6
Group-3	0	← Less than 1 →					
	0						
	0						
Mean	0						

-Group-1= vaccinated with rabies vaccine alone.
-Group-2= vaccinated with rabies vaccine and BCG.
-Group-3 = non vaccinated control.
*WPV= week post vaccination **MPV= month post vaccination.

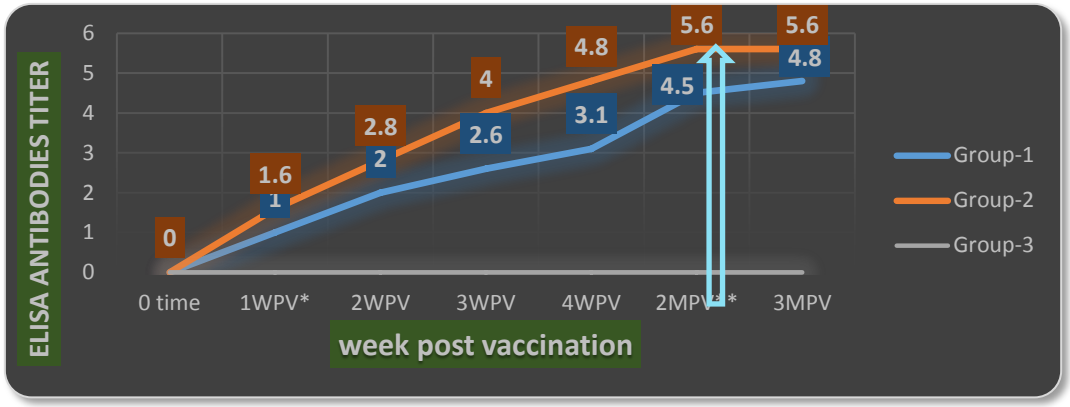

Figure (2) Rabies ELISA antibody titers in experimentally vaccinated puppies.

-Group-1= vaccinated with rabies vaccine alone.
-Group-2= vaccinated with rabies vaccine and BCG.
-Group-3 = non vaccinated control.

Apparently, The outcomes of the ELISA kit test were similar to those of SNT and Indirect ELISA confirming that BCG inoculated simultaneously with rabies vaccine induced higher protection percentage (38.3% by the first week and 90% by the second month post vaccination) than that induced by rabies vaccine alone (33.3% and 85% by the first week and second month post vaccination respectively) as seen in table (3) and figure (3).

Table (3): Rabies protection % in experimentally vaccinated puppies as detected by ELISA kit.

Puppy groups	Rabies protection %						
	0 time	1WPV*	2WPV	3WPV	4WPV	2MPV**	3MPV
Group-1	0	30	40	60	80	85	85
	0	35	45	65	70	85	85
	0	35	50	70	80	85	85
Mean	0	33.3	45	65	76.8	85	85
Group-2	0	35	70	75	80	90	90
	0	40	75	80	85	90	90
	0	40	80	85	90	90	90
Mean	0	38.3	75	80	85	90	90
Group-3	0	← 0 →					
	0						
	0						
Mean	0						

-Group-1= vaccinated with rabies vaccine alone.
-Group-2= vaccinated with rabies vaccine and BCG.
-Group-3 = non vaccinated control.
*WPV= week post vaccination **MPV= month post vaccination
-ELISA PI% is approximately 60% of 0.5 IU/ML

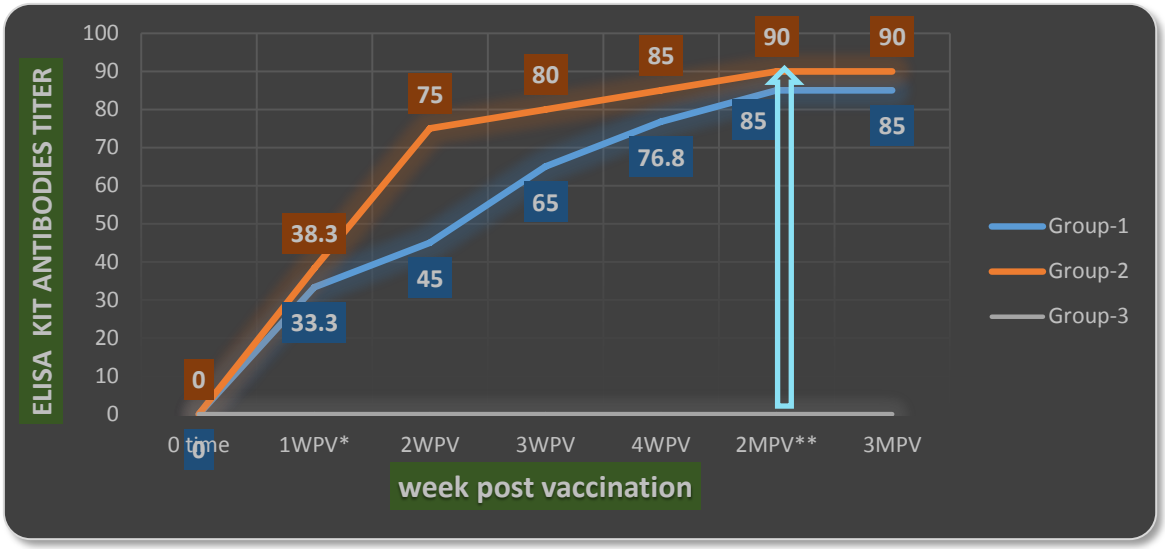

Figure (3): Rabies protection % in experimentally vaccinated puppies as detected by ELISA kit.

-Group-1= vaccinated with rabies vaccine alone.

-Group-2= vaccinated with rabies vaccine and BCG.

-Group-3 = non vaccinated control.

Latex agglutination test (LAT) was found to be able to detect rabies antibodies in the sera of vaccinated puppies with clear strong agglutinations with high antibody concentrations started from the 3rd week up to the 3rd month post vaccination while weak positive agglutinations were observed during the first 2 weeks post vaccination. The test also confirmed the results of SNT; indirect ELISA and ELISA kit. LAT results are tabulated in table (4).

Table (4): Detected Rabies antibody titers in experimentally vaccinated puppies by latex agglutination test.

Puppy groups	Dilution of positive sera (tenfold dilution)						
	0 time	1WPV*	2WPV	3WPV	4WPV	2MPV**	3MPV
Group-1	-	With all tested serum samples obtained from experimentally vaccinated puppies, LAT showed clear strong positive agglutination high antibody titers (from the 3rd week to the 3rd month post vaccination). Weak agglutinations were observed through the first week's					
	-						
	-						
Group-2	-						
	-						
	-						
Group-3	-	← Negative all over the experimental period→					
	-						
	-						

-Group-1= vaccinated with rabies vaccine alone.
-Group-2= vaccinated with rabies vaccine and BCG.
-Group-3 = non vaccinated control.
*WPV= week post vaccination **MPV= month post vaccination

Monitoring the amount of rabies antibodies in the serum of vaccinated puppies through application of indirect FAT also confirmed that puppy's inoculation with BCG simultaneously with rabies vaccine resulted in earlier and higher levels of rabies antibodies than those resulted by rabies vaccine alone showing positive reactions up to dilutions of 6log10 and 5log10 respectively by the second month post vaccination as shown in table (5) and figure (4).

Table (5): Rabies antibody titers detected by indirect FAT in the sera of experimentally vaccinated puppies.

Puppy groups	Dilution of positive sera (tenfold dilution)						
	0 time	1WPV*	2WPV	3WPV	4WPV	2MPV**	3MPV
	-	1	2	3	4	5	5
	-	1	2	3	4	5	5
Group-1	-	1	2	3	4	5	5
Mean	-	1	2	3	4	5	5
	-	2	3	4	5	6	6
	-	2	3	4	5	6	6
Group-2	-	2	3	5	5	6	6
Mean	-	2	3	4.5	5	6	6
	-	← Negative all over the experimental period→					
	-						
Group-3	-						
Mean	-						

-Group-1= vaccinated with rabies vaccine alone.
-Group-2= vaccinated with rabies vaccine and BCG.
-Group-3 = non vaccinated control.
*WPV= week post vaccination **MPV= month post vaccination

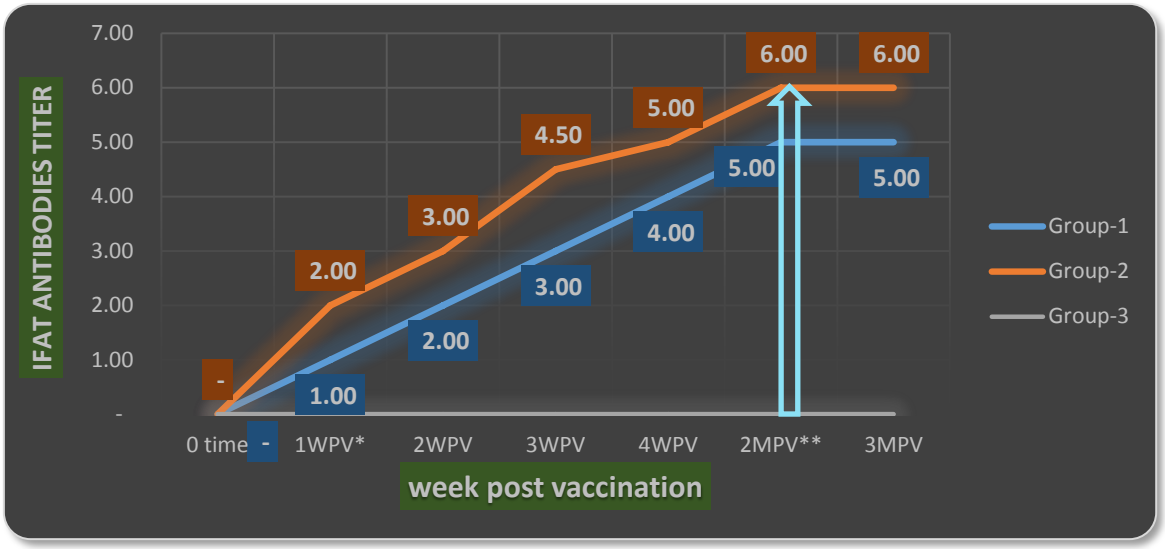

Figure (4) Rabies antibody titers detected by indirect FAT in the sera of experimentally vaccinated puppies

-Group-1= vaccinated with rabies vaccine alone.

-Group-2= vaccinated with rabies vaccine and BCG.

-Group-3 = non vaccinated control.

4. Discussion

The current study was conducted on two puppy groups, one vaccinated with Carbopol adjuvanted inactivated rabies vaccine alone and the other with Rabies and BCG vaccine, leaving a third group without vaccination as the test control. The study's purpose was to assess the immunological response of puppies injected with the local inactivated rabies vaccine alone and with an immune stimulating drug. The serum neutralisation test (SNT), indirect enzyme linked immunosorbent assay (ELISA), indirect fluorescent antibody technique (IFAT), rabies virus antibody quantitate ELISA kit, and latex agglutination test (LAT) were all used to monitor the induced rabies antibodies in the sera of puppies who had received the vaccine.

It was found that puppies who received the rabies and BCG vaccines at the same time developed serum neutralizing antibodies more quickly and at greater levels (mean values of 5.3 and 2,6 by the first post-vaccination week, and male values of 106.6 and 53.3, respectively, by the second post-vaccination month). According to the SNT results (table 1 and fig. 1), rabies serum neutralizing antibody titers of >16 were classed as positive **(Vos *et al.*, 2001).** Our current findings matched and were validated by the findings of who found similar results and indicated that the cell culture inactivated rabies vaccine is safe for all animal species and highlighted that the protective neutralizing antibody titer should be more than 1:5 (**Sikes *et al.*, 1971; Bass *et al.*, 1982; Khodier, 1999; Khodeir and Daoud, 2008 and Albehwar, 2009)**

Parallel to the results of SNT and ELISA indicated that simultaneous injection of BCG with rabies vaccine exhibited vaccinated puppies with higher levels of rabies antibodies than those in single rabies vaccinated ones (1.6 and 1 by the first week and 5.6 and 4.5log10 by the second month post vaccination respectively) (table-2 &fig.2).

Additionally, the current ELISA kit results (table 3 and fig. 3) demonstrated greater protection with simultaneous BCG and rabies vaccination compared with a single rabies vaccination (90 and 85% protection). In this regard, a positive blocking rate (PI%) of 60%, or 0.5 IU/ML, was suggested as being enough for defending against rabies infection in dogs who had received the vaccine. Additionally, it was discovered that vaccinated foxes had titers of less than 0.5 IU/ml and could withstand a challenge, but all foxes with titers higher than 0.5 IU/ml died from rabies **(Müller *et al*., 2001)**.

On the other hand, the results of LAT and indirect FAT paralleled and supported those of SNT, indirect ELISA, and ELISA kit, demonstrating that BCG enhances the immune response of puppies who have received the rabies vaccine, leading to high levels of specific rabies immunity. The serum neutralizing test is used as the bioassay method of choice for evaluating the acceptability of horse antisera and is preferred by the majority of laboratories for determining rabies antibody levels in rabies-vaccinated puppies **(Smith *et al.*, 1973).**

To measure the amount of rabies antibodies, an enzyme-linked immunosorbent test (ELISA) has been developed **(Nicholson and Prestage, 1982; Kavaklova *et al.*, 1984; Grassi *et al.*, 1989).**

A straightforward rabid test for detecting and measuring rabies antibodies is the use of an ELISA kit **(Bio-Equip, 2020).**

A latex agglutination test (LAT) has been shown to be useful for detecting rabies antibodies following human immunization. The LAT is simple to perform and needs minimum laboratory equipment (**Perrin *et al.*, 1988; Madhusudana and Saraswati, 2003).**

The FAT has a sensitivity of 99.78% (**Bryceson *et al.*, 1975).** IFAT was more sensitive than ELISA for detecting subclinical infection (75 vs 70%), while ELISA was superior for diagnosing clinical leishmaniosis (98 vs 97%) **(Maria *et al.*, 2017).**

Concerning the use of BCG alongside rabies vaccine, there is some evidence that BCG vaccination increases the humoral immune response to unrelated immunizations, effectively serving as an adjuvant **(Ota *et al.*, 2002; Mackaness, 1968 and Soliman *et al*, 2011).** Furthermore, BCG immunization given concurrently with oral polio vaccine boosted antibody response to polio, confirming BCG's systemic effect **(Rossenthal, 1980).**

Our current findings show that concomitant vaccination of puppies with rabies and BCG vaccines elicited high particular rabies antibody levels as assessed by all serological tests. BCG sensitized animals where macrophages charged rapidly;

processes lysosomal and mitochondria enzymes. Furthermore, BCG was recognized as a non-specific immune activating agent.

5. Conclusion

Based on the current findings, it is possible to assume that BCG vaccination boosts the immunological response of vaccinated puppies, resulting in high levels of particular rabies antibodies. Furthermore, SNT, indirect ELISA, quick ELISA kit, LAT, and IFAT can detect rabies antibodies in the sera of vaccinated puppies.

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Author contributions:

All authors contributed to administrative support, study material provision, and final manuscript approval, as well as data collection and assembly, data analysis and interpretation, and paper writing. The final manuscript was read and approved by all of the authors.

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Availability of data and materials:

This published article contains all of the data gathered and analysed throughout the experiment.

Ethical approval

The National Research Centre in Egypt's Medical and Veterinary Research Ethics Committee gave its blessing to the treatment and usage of the animals (No. 20/053).

Conflict of interest:

The authors declare that they have no competing interests.

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تتبع الحالة المناعية للسعار فى الأجراو المحصنة تجريبيا

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من المعروف ان مرض السعار هو مرض فيروسى مميت ويتسبب فى مخاطر عاليه فى كل من الانسان والحيوان. وعلى ذلك فلابد من السيطرة عليه فى العائل الاساسى للمرض وخاصه الكلاب وذلك بالتحصين بلقاحات عاليه الكفاءه وتحفيز استجابتهم المناعيه لاكسابهم مناعه عاليه ضد المرض.

وقد تم خلال العمل الحالى تحصين مجموعات مختلفة من الأجراو بلقاح السعار المثبط المنتج محليا فقط ومع لقاح البى سى جى ثم تتبع المستويات المناعية المحدثة فى أمصالها بتطبيق بعض الاختبارات السيرولوجية حيث أوضحت نتائج أختبار المصل المتعادل أظهر أن ال بى سى جى أحدث مستويات عاليه ومبكره من الاجسام المناعيه بمتوسط قيمته ١٠٦.٦ فى نهايه الشهر الثانى بعد التحصين بلقاحى السعار وال بى سى جى فى نفس الوقت ولكن عند حقن لقاح السعار منفردا كان المستوى ٥٣.٣ .

كما أظهر أيضا اختبار الانزيم المرتبط المناعى الممدص الغير مباشر مستوى مناعى أعلى فى الأجراو المحصنة بلقاحى السعار وال بى سى جى(٦لوج١٠) منه فى الأجراو المحصنة بلقاح السعار فقط (٤.٥ لوج ١٠) وذلك بعد شهرين من التحصين. كما جاءت نتائج كاشف الانزيم المرتبط المناعى مؤكده أن لقاح السعار مع البى سى جى أحدث استجابه مناعيه أعلى(٩٠%) من التى أحدثها لقاح السعار منفردا (٨٥%) وذلك بعد الاسبوع الاول والشهر الثانى من التحصين. كما أظهر اختبار اللاتكس التلزنى وجود اجسام مناعيه عاليه ضد مرض السعار فى أمصالالأجراوالمحصنه بينما أظهر مستوى منخفض من هذه الاجسام المناعيه خلال الاسابيع الاولى والاخيره بعد التحصين ويأتى هذا بالتوازى مع نتائج أختبار المصل المتعادل والانزيم المرتبط المناعى الممدص الغير مباشر وكاشف الاليزا٠١ وعلى الجانب الآخر أكد أختبار الوميض الفلوريسنتى المناعى الغير مباشر أن التحصين المتزامن بلقاح السعار وال بى سى جى يحدث مستويات من الاجسام المناعيه المبكره والعاليه عن التى يحدثها لقاح السعار منفردا وذلك بتخفيفات ٦ لوج ١٠ و ٥ لوج ١٠ على الترتيب بعد الشهر الثانى من التحصين.

وبناءا على النتائج العملية لهذه الدرساة فإنه يمكن القول بأن ال بى سى جى يمكن أن يستخدم كمحفز مناعى لتحسين الاستجابه المناعيه للكلاب المحصنه بلقاح السعار كما أن نذكر كل الاختبارات السيرولوجيه المستخدمه قادره على الكشف عن الاجسام المناعيه فى أمصال الحيوانات المحصنه كميا.

الكلمات المفتاحية: داء السعار- أختبار المصل المتعادل - أختبار اللاتكس التلزنى - أختبار الوميض الفلوريسنتى المناعى الغير مباشر.