

# Detection of Neutralising Antibody Titration in Vaccinated Owned and Stray Dogs against Rabies Virus<sup>#</sup>

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## ABSTRACT

The aim of this study was to determine the titers of neutralising antibodies against rabies virus in five hundred vaccinated owned and stray dogs within the İstanbul province. The levels of the neutralising antibody titration in the blood serum were evaluated by Enzyme-Linked Immunosorbent Assay (ELISA) method. Sixty five (13%) of the dogs examined had adequate level (0.5 IU/ml and over) of antibody titration. Also, the effects on the formation of the neutralising antibody levels following the vaccination of some factors, such as being owned or stray, the number of repetition of vaccination, the age, living area, sex, interval between the last vaccination and sampling, have been evaluated statistically. As a result of the statistical evaluations, it has been determined that interval between the last vaccination and sampling had an important effect on formation of the neutralising antibody titration ( $P<0.001$ ). Also, it was determined that the effect of the age of the dog was also significant for the neutralising antibody levels ( $P<0.05$ ). On contrary, being owned or stray, sex, living area and the number of repetition of the vaccination had no effects on the neutralising antibody levels.

**Key Words:** Dogs, rabies, vaccines, immunogenicity of rabies vaccine

## ÖZET

### AŞILANMIŞ SAHİPLİ VE SAHİPSİZ KÖPEKLERDE KUDUZ VİRUSUNA KARŞI OLUŞMUŞ NÖTRALİZAN ANTİKOR SEVİYELERİNİN SAPTANMASI

Bu çalışmada, İstanbul İli sınırları içerisinde yaşayan, aşılanmış sahipli ve sahipsiz beşyüz köpekte kuduz virusuna karşı oluşmuş nötralizan antikor seviyelerinin saptanması amaçlandı. Kan serumlarında oluşmuş nötralizan antikor titreleri ELISA ile araştırıldı. İncelenen köpeklerin 65 tanesi (%13) yeterli düzeyde nötralizan antikor (0,5 IU/ml ve üzeri) titresine sahipti. Ayrıca aşılamayı takiben oluşmuş nötralizan antikor seviyelerine sahiplilik durumu, aşı tekrar sayısı, yaş, yaşam alanı, cinsiyet, son aşı ile örnekleme arası geçen süre gibi faktörlerin etkileri istatistiksel değerlendirme ile incelendi. İstatistiksel değerlendirmeler sonucunda, son aşı ile örnekleme arası geçen sürenin şekillenmiş nötralizan antikor titelerine etkisinin önemli olduğu ( $P<0,001$ ) belirlendi. Ayrıca köpek yaşlarının da nötralizan antikor seviyeleri için önemli olduğu saptandı ( $P<0,05$ ). Buna karşın, sahiplilik durumu, cinsiyet, yaşam alanları ve aşı tekrar sayısının oluşmuş nötralizan antikor seviyeleri üzerine etkili olmadığı belirlendi.

**Anahtar Kelimeler:** Köpek, kuduz, aşı, kuduz aşısına karşı bağışıklık

<sup>#</sup> This study was summarised from first author's PhD thesis.

### Introduction

The rabies is an important viral zoonotic disease that can be seen in all warm blooded animals and human beings and can cause death by affecting the Central Nervous System (Doymaz, 2005; Johnson, 1965; Ruppercht et al., 2002). As reported by the World Health Organisation (WHO), every year, nearly 55,000 people are losing their lives because of rabies (WHO, 2010). According to the WHO reports, a total of 301 rabies cases were reported in Turkey, in 2008, 257 of which were domestic and 44 were wild animals. Of these rabies cases reported, 146 (49%) were dogs, 85 (28%) were cattle and 70 (23%) were other animals (WHO, 2008). In the first quarter of 2009, 64 cases were reported 2 of which were wild animals and 62 were domestic animals. And reported cases have shown a decrease of 33% when compared to the same period of the previous year (WHO, 2009).

In the 2010 version of the "Contestation Program with Animal Diseases and Injurious" which is prepared by Ministry of Agriculture and Rural Affairs (MARA), General Directorate of Protection and Control every year, it has been stated that the control of reproduction of stray dogs is important for the control of rabies. On the control side, it has been expressed that the local administrations are responsible for eliminating the living areas (especially the refuse dumps) of the stray dogs, establishing observation places with enough accoutrements and capacity, following up the vaccinations carrying out by independent Veterinary Surgeon and voluntary organisations, collection, castrating and identification of stray animals (MARA, 2010).

According to WHO, for the vaccinated dogs to be accepted as immune, they must have a minimum 0.5 IU/ml neutralising antibody titration (WHO, 1992; WHO, 2004). European Union Commission (EU) also took some protective measures for the animals that have possibility of carrying rabies, by implementing new laws regarding this issue (EC, 2003). This law was prepared especially for the animals that are travelling frequently within EU and has

been put into force since October 1<sup>st</sup>, 2005 in Turkey too. According to the last decision taken by the Ministry of Agriculture and Rural Affairs, in parallel with the EU applications, for the dogs, cats and ferrets to travel to EU countries (other than UK, Malta, Sweden and Ireland) it is expected for those animals to be clearly identifiable by a tattoo or an electronic identification system. Also, it must be verified by an authorised veterinary physician that in a sample blood taken earliest 30 days after the last vaccination made and minimum three months before departing from the origin country, the level of the neutralising antibody titration is equal to or higher than 0.5 IU/ml (MARA, 2010).

Affection towards animals is increasing as the development level of the societies increase and in parallel, the number of people having pets also shows an increase. The number of stray animals in our country is also undeniably high. Dogs that are vaccinated by various campaigns that are taking part in our social lives with a ring in their ears and are assumed to be protected against rabies are present, especially in Istanbul, in large numbers. But, in Turkey, the formation of the necessary immunisation is never checked regularly. For the reasons stated above, in this study, it was aimed to determine the immunisation level formed in the owned and stray dogs that have been vaccinated against rabies.

### Materials and Method

#### Samples

Between May 2004 and March 2005, samples were taken from five hundred dogs within the city limits of Istanbul. Of the blood samples, 170 were taken from stray and 330 were taken from owned dogs. Sex, ages, living areas, number of vaccination repetition and the latest vaccination dates of the dogs were noted. When samples were taken from the stray dogs, care was taken to collect samples from the dogs with earrings which indicate that they were vaccinated. Sex, age of some and their dates of vaccination were noted. For the stray dogs it was not possible to determine their number of vaccination repetition.

**Sex:** 250 of the dogs were female and 250 were males.

**Living Areas:** It was determined that 213 of the owned animals lived in the houses and 117 lived in the gardens.

**Age:** It was determined that the age of the owned dogs changed between five months and 19 years. Of these dogs, 40 were in five months-one year age group, 104 were in two-five years, 81 in the six-nine years and 105 were in the 10 years and over age group. The ages of 48 out of 170 stray dogs were determined, of these it was seen that their ages ranged between five months and 12 years. Of these dogs, nine were in five months-one year age group, 27 were in two-five years, eight in six-nine years and four were in 10 years and over age group.

**Number of Repetition of Vaccination:** The numbers of vaccination repetition were determined for the owned dogs, but it was not possible for the stray dogs. But, from their earrings, it was understood that they were vaccinated at least once. Accordingly, 57 of the dogs were only vaccinated once, 30 were vaccinated twice, 20 were vaccinated three times, 37 were vaccinated four times, 54 were vaccinated five times, 58 were vaccinated six times, 47 were vaccinated seven times and the remaining 27 were vaccinated 10+ times.

**Intervals between the Last Vaccination and Sampling:** Intervals between the last vaccination and the sampling dates were determined for the owned dogs. But, for the stray dogs, it was only possible to determine these periods for the 65 of the stray dogs.

### **Serology**

For the purpose of determining the formation of the neutralising antibodies in the dogs serum, MCA based blocking ELISA kit (BV European Veterinary Laboratory EVL, The Netherlands) was used.

The test was applied as to the method that is specified in the kit. The ELISA results were evaluated by reading with Organon Technica Microwell System Reader 230 optical reader at 450 nm wavelength. The qualitative evaluations

of the tests were made by using the evaluation criteria given in the kit.

### **Statistical Evaluation**

The effects on the neutralising antibody formation levels of being owned or stray, sex, number of repetition of vaccination and interval between the last vaccination and sampling date have been evaluated. In the comparison of being owned or stray, sex and living areas of the owned dogs "Independent Sample T-Test" was used (Özdamar, 1999). In the comparison of being a owned or stray 330 owned and 170 strays, in comparison of sex 250 females and 250 males, in comparison of the living areas of the owned dogs 117 in-garden and 213 in-house dogs were evaluated using independent sampling T-test.

The statistical comparisons of the ages, number of repetition of vaccination, interval between the last vaccination and the sampling date groups were made by using "One Way Variance Analysis" (F Test, Anova) (Özdamar, 1999). When the difference in the criteria used for one way variant analysis was significant, then Duncan Method was utilised for determining the importance among which groups (Özdamar, 1999).

In examining the importance of age in formation of the sufficient level of neutralising antibody formation by one way variant analysis, four groups were formed for 1 year and under (n=49), 2-5 years (n=131), 6-9 years (n=89) and 10 years and over (n=109). Numbers of samples per group were also determined (Table 1).

In evaluation of the number of repetition of the vaccination by one way variant analysis, eight groups were formed, once (n=57), twice (n=30), three times (n=20), four times (n=37), five times (n=54), six times (n=58), seven-nine times (n=47), 10 times and over (n=27) (Table 2).

In the evaluation of the interval between the last vaccination and the sampling by one way variant analysis 11 groups were formed. Table 3 shows the numbers of samples per periods (Table 3).

**Tablo1.** Yaş grupları.  
**Table 1.** Age groups.

Group No	Age Groups (Years)	No. of Samples
1	5 months – 1 years	49
2	2-5	131
3	6-9	89
4	10+	109

**Table 2.** Number of repetition of vaccination groups.  
**Tablo 2.** Gruplardaki aşılama sayısı.

Group No	No. of Repetition of Vaccination	No. of Samples
1	1	57
2	2	30
3	3	20
4	4	37
5	5	54
6	6	58
7	7-9	47
8	10+	27

**Table 3.** Interval between the last vaccination and sampling  
**Tablo 3.** Son aşılama ve örnekleme arasında geçen süre

Group No.	Interval between the Last Vaccination and Sampling Date (Month)	No. of Samples
1	15 days - 2 months	50
2	3-4	34
3	5-6	38
4	7-8	76
5	9-10	22
6	11-12	37
7	13-24	37
8	25-36	15
9	37-60	33
10	61-84	31
11	85 +	22

## Results

### ELISA Results

Results of the ELISA have shown that 65 sera had adequate titers ( $\geq 0.5$  IU/ml) of neutralising antibodies. Of these 65 sera, 46 belonged to the owned dogs and 19 belonged to the stray dogs. Of the 46 owned dogs, 12 lived in the garden and 34 lived in the houses. Of the

positive serums, 35 serums belonged to males and 30 belonged to females.

Of the owned dogs with adequate level of antibody titrations, it has been established in the age distribution that 10 were in the five months-one year age group, 17 were in the two-five years age group, 12 were in the six-nine years age group and seven were in the 10 years and

over age group. Of the stray dogs with adequate level of antibody titrations, it has been established that one was in the five months-one year age group and four were in the two-five years age group, for the other 14, age groups could not be determined (Table 4).

When the number of repetition of the vaccination of the positive animals, it was established that out of the 46 pets, 13 was

vaccinated once, five were vaccinated twice, one was vaccinated 3 times, seven were vaccinated 4 times, eight were vaccinated 5 times, five were vaccinated 6 times, three were vaccinated 7-9 times and four were vaccinated 10 times and more. Of the 19 stray animals, it was not possible to establish the number of repetition of vaccination (Table 5).

**Table 4.** Yaş grupları.

**Table 4.** Age groups.

Age Group (Years)	No. of Samples	No. of Positive Samples
5 months - 1 year	49	11
2-5	131	21
6-9	89	12
10+	109	7

**Table 5.** Number of repetition of vaccination

**Table 5.** Aşılama tekrar sayıları.

Number of Repetition of Vaccination	No. of Samples	No. of Positive Samples
1	57	13
2	30	5
3	20	1
4	37	7
5	54	8
6	58	5
7-9	47	3
10+	27	4

When interval between the last vaccination and the sampling date was examined, it was established that in these animals interval was

between 15 days and 48 months. In 14 of the stray animals, this information could not be obtained (Table 6).

**Table 6.** Interval between the last vaccination and sampling.

**Table 6.** Son aşılama ve ömikleme arasında geçen süre.

Interval Between the Last Vaccination and Sampling (Month)	No. of Samples	No. of Positive Samples
15 days – 2 months	50	9
3-4	34	4
5-6	38	7
7-8	76	7
9-10	22	5
11-12	37	6
13-24	37	7
25-36	15	2
37-60	33	2
61-84	31	2
85 +	22	-

### Results of statistical evaluations

It has been established as a result of the independent sample t-test that being owned or stray, sex and living area of the owned dogs had no effects on the formation of the neutralising antibody levels.

When the ages of the dogs were evaluated by the one way variant analysis, it has been understood that there was a significant difference ( $P < 0.05$ ). Of the groups formed, when Duncan test was applied to determine the significant between the groups, it has been understood that the difference of first group with third and fourth groups, and the difference of the fourth group with first and third groups were significant.

As a result of the one way variant analysis, it has been found that the number of repetition of vaccination was significant for the level of the neutralising antibody formation ( $P < 0.05$ ).

When one way variat analysis was applied, it was seen in the results that interval between the last vaccination and sampling date had very significant effects on the formation of neutralising antibody titers ( $P < 0.001$ ). As the result of the Duncan test that applied to determine the importance relations between the groups, it has been established that the difference of the 10<sup>th</sup> and 11<sup>th</sup> group with the first, second, third, fifth, sixth and seventh groups were significant. Also, the difference of the sixth group with eighth and ninth groups were determined to be significant.

### Discussion

WHO has reported that the minimum level of neutralising antibody titers in the serum for protecting against rabies to be 0.5 IU/ml (WHO 1992, WHO 2004).

Cliquet et al. (2003) have used Rapid Fluorecent Focus Inhibition Test (RFFIT) and Fluorecent Antibody Virus Neutralisation Test (FAVN) on the samples taken from 17693 vaccinated dogs for determining neutralising antibodies and established that 92.6% of the dogs had adequate ( $\geq 0.5$  IU/ml) neutralising antibodies. Fooks et al. (2002), on a study they

carried out on blood samples taken from 20597 dogs, determined the neutralising antibodies by FAVN test and reported that 98.7% of the animals had adequate titers. Mansfield et al. (2004), between 1999 and 2002 determined the neutralising antibody titers on 14035 dogs by FAVN test and reported that on the samples studied twice, 95.88% and 94.85% of them had adequate levels of antibodies. Sugiyama et al. (1997) determined the neutralising titers on the serums of 1019 vaccinated dogs by Competitive Enzyme-Linked Immunosorbent Assay (c-ELISA) test and reported that 84.4% had adequate antibody titers. Delgado and Carmenes (1997), examined 156 serum samples taken from owned dogs by ELISA and reported that 58.3% carried sufficient level of neutralising antibody but stressed that since the results belonged to owned dogs, did not reflect the correct sero - prevalence. Sage et al. (1993), in the study they carried out by RFFIT method for determining the antibody titrations in vaccinated animals have established that in the second month after the vaccination 27%, in the sixth month 24% and in the first year 33% of the animals had below 0.5 IU/ml antibody titration level. Kasempimolporn et al. (2007), in their study on 3314 vaccinated stray dogs determined neutralising antibodies using ELISA and reported that in 62% of the titers were insufficient.

In this study, vaccinated dogs were evaluated using ELISA kit for determination of neutralising antibodies titers formed against rabies virus and it was established that the sufficient neutralising antibody titers were at a low level (13%) when compared to the other results announced. It was thought that the low level could have been due to stray dogs, but according to the results of statistical analysis made, it was seen that being a owned or a stray dog had no effect on the antibody titrations formed.

Thinking that the number of repetition of the vaccination could have affected the antibody titers formed against the rabies virus, in a study carried out by Mansfield et al. (2004) they have observed that after the second dose, antibody titers increased a great deal and reported that

vaccination should not be applied in a single dose and each vaccination should have meant two doses. Shimazaki et al. (2003) in a study they carried out on 87 puppy dogs have determined that 1 month after the vaccination, the level of antibody titers that rose to 1.0-2.0 IU/ml, fell down to 0.2-1.5 IU/ml level after 1 year but after second vaccination titrations showed a considerable increase and rose to a level between 12 and 47 IU/ml. The researchers concluded that the low levels of the antibody titrations were due to maternal antibodies carried by the dogs and single dose application of the vaccination Shimazaki et al., (2003). Cliquet et al. (2003) in their study carried out using 17693 dog serum, they have reported that the dogs vaccinated twice or more carried considerably high rate of neutralising antibodies, when compared to those vaccinated only once. They have also reported that twice or more vaccinations did not have a significant titers difference.

But in this study, after the statistical analyses made on the sera samples of 330 owned dogs whose number of repetition of vaccinations were known, for the effect of vaccination repetition number, it was established that this had an insignificant effect on the final determination of the antibody titers. With this result contradicted with the results from other researches, it was thought that the reason was probably because of the low percentage of the immune animals determined during the studies.

The researchers have expressed that the age had a significant effect on the immune response, especially those that are under 1 year old and those that are old (Mansfield et al., 2004). Hogenesch et al. (2003) in an experimental study they have carried out on two groups of dogs, one old (12 years) with higher level of antibody titers and the other young (3 years) with lower titer levels to show the effect of age, have established after repeating vaccinations on both groups that the levels of antibody titers in the younger animals have reached the levels of old animals and a better immune response was formed in the young ones. Delgado and Carmenes (1997) have

reported after studying the serums they received from 156 pets from both two regions that the age did not have an effect on the antibody levels formed.

In this study, it was observed that age had an effect on the antibody titrations formed and the neutralising antibody titers formation in the old animals were considerably lower when compared to the other age groups. This condition which is in line with the other researchers is connected to the reduction of immunisation capability in the old animals.

Delgado and Carmenes (1997), when they compared the neutralising antibody levels of 71 dogs living in houses, 56 dogs living in gardens and 29 dogs living in dog breeding places, have reported no difference between the groups.

In this study, it was determined that among 330 owned animals, 213 living in houses and 117 living in the gardens; the neutralising antibody titers formation was not affected by the living area.

Verthely and Klinman (2000) have reported that another factor that could have affected the antibody levels could have been sex and that the sex hormones could have formed the production of cytokines and that in turn would have caused some changes in immune response. But, in various other studies, the researchers have announced that sex did not have any effect on the antibody levels formed against rabies virus (Delgado and Carmenes, 1997; WHO 1998; Mansfield et al. 2004). Delgado and Carmenes (1997) have announced after their study on 79 male and 77 female dogs that sex was ineffective. Mansfield et al. (2004), in a study where they determined neutralising antibody titers on vaccinated cats and dogs, have reported that while sex had an effect for the cats, had no effect for the dogs.

This study shows similarity with the results that announce that neutralising antibody titers have no relations with sex.

The researchers have reported that antibody titers is a curve decreasing in time after the rabies vaccination and have stressed that interval between vaccination and sampling is an

significant factor that effects the neutralising antibody titers formed (WHO 1998, Fooks et al., 2002; Cliquet et al., 2003; Mansfield et al., 2004). Cliquet et al. (2003) have observed that when interval between vaccination and sampling was 2 months, 7.8%, when it was 4 months 19.1% and when 6 months 25% of the samples have shown insufficient levels of neutralising antibodies and as interval between vaccination and sampling increased, the rate of negativity increased. In another study, while seropositivity was detected at a ratio of 90% on the 22<sup>nd</sup> day, 5 months later this ratio was observed to drop down to 71.1% (WHO, 1998).

In this study, in the statistical analysis made on 395 samples where interval between the vaccination and sampling was known, it has been observed that interval between vaccination and sampling was important. In parallel to the other studies, it has been determined that for a period of two years or more, the levels of neutralising antibody titers were lower.

The researchers have also stressed that besides the personal effects, preparation; transporting, storing and application of the vaccines play an important role effectiveness of the vaccine (WHO, 1999; Lloyd, 2000; Lala and Lala 2003). For the required immune level to be reached, it was reported that the vaccine should have been carried in cold chain (WHO, 1999).

The cold chain is described as all necessary vehicles, equipment and people for storing, distributing and transportation of the vaccine, from the production plant to the application point (Lloyd, 2000; Lala and Lala, 2003; WHO, 2006). The researchers also stress that depots, coolers, isothermal boxes, portable ice boxes as well as warehouse workers and vaccine applicators are a part of the cold chain (ATAG, 2003; Lala and Lala, 2003).

Kıyan (2001), in a study he carried out with the participation of 105 health personnel working in the general health centres, mother-child health centres, family planning centers and tuberculosis combat centres, on suitability

of the coolers and vaccines and sufficiency of the health personnel on knowledge of cold chain and the existing conditions were found inadequate for providing cold chain.

Bozkaya (2000), in a study he carried out in 23 medicine depots selling biological products, for the suitability of the conditions and providing and continuing cold chain, has observed that the personnel did not have sufficient knowledge on the characteristics of the equipment, working systems, providing and continuing ideal areas for storage. When it is considered that it is compulsory that all the conditions must be complied with in order to provide a cold chain properly, it has been reported that none of the depots could comply with all the requirements.

In this study, when the antibody titers of owned dogs that are vaccinated regularly are examined, it has been determined that 14% did not have the required neutralising antibody level. This low level of positive ratio suggests that there was a different factor other than those examined, which is affecting these values.

As a result, majority of the owned and stray dogs that have been vaccinated for rabies did not have the sufficient levels of neutralising antibody titers. The dogs which are the major causes for human rabies, having believed that they are safe since they have been vaccinated, have low levels of antibodies and are carrying great risks for the human beings. For the local administrations that are responsible of combating with rabies in the dogs, an example of an vaccination campaign must be started nationwide. In order to identify the vaccinated animals, a single type of identifier must be used nationwide, with documentary facilities.

The cold chain for the vaccines in line with the directives of the manufacturers from production line to medicine depots and veterinary offices and animal farms where the final applications are made, controls must be made during storage and transportation. Also, the related personnel on these points must be trained for protection and continuity of the cold chains.



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