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Original article

Vaccination of Tunisian dogs with the lyophilised SAG2 oral rabies vaccine incorporated into the DBL2 dog bait

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Abstract – The protective effect of the lyophilised SAG2 oral vaccine bait DBL2, already demonstrated on laboratory dogs, needed to be verified on common Tunisian dogs. Seven Tunisian dogs consumed totally or partially one DBL2 bait containing $10^{8.3}$ TCID₅₀ of the highly attenuated rabies vaccine strain, SAG2. Five of the seven vaccinated animals survived a challenge administered 33 days later with a Tunisian canine street rabies virus to which five of the six controls that were not vaccinated and had no specific antibodies succumbed. The partial or total consumption of a single DBL2 bait thus conferred a protective immune response similar to that observed in laboratory dogs to dogs of poor health status. The sero-antibody response was, however, weak: only two vaccinated dogs exhibited a significant neutralising antibody response after vaccination and before the challenge, and four after the challenge. © Inra/Elsevier, Paris.

rabies / dog / lyophilised oral vaccine

Résumé – Vaccination de chiens tunisiens à l'aide de la souche rabique SAG2 lyophilisée (appât DBL2). La protection conférée par l'appât lyophilisé DBL2 contenant la souche hautement atténuée DBL2, bien que démontrée sur chiens de laboratoire devait être vérifiée sur chiens tout venant tunisiens. Sept de ces chiens ont consommé partiellement ou en totalité un appât lyophilisé DBL2 contenant $10^{8.3}$ DICT₅₀ de la souche SAG2. Cinq des sept chiens vaccinés ont survécu à une épreuve virulente administrée 33 j plus tard avec une souche de rage canine locale qui a entraîné la mort de cinq des six chiens témoins non vaccinés et ne possédant pas d'anticorps spécifiques avant épreuve.

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La consommation totale ou partielle d'un seul appât DBL2 contenant la souche SAG2 confère donc à des chiens de statut sanitaire déficient une protection comparable à celle qui a été observée chez des chiens de laboratoire. Cependant la production d'anticorps séroneutralisants a été faible : seulement deux chiens vaccinés ont présenté des anticorps à un titre significatif après vaccination et avant épreuve, et quatre chiens au total après épreuve. © Inra/Elsevier, Paris.

rage / chien / vaccin oral lyophilisé

1. INTRODUCTION

Canine rabies remains a serious public health problem in Tunisia despite a national control programme initiated in 1982 consisting of the epidemiosurveillance of the disease in animals, mass campaigns of parenteral vaccination of dogs, the elimination of stray dogs and public education.

These measures, in particular the annual vaccination of dogs throughout the whole country, significantly decreased rabies cases in animals and concurrently in humans for several years. In 1985 and 1988, the number of animal cases was limited to 60 and 48, respectively, and no human rabies cases were recorded [5]. Unfortunately, these promising results were not repeated the following years: rabies is still present in the dog population and human deaths are recorded every year (up to 25 in 1992).

One of the possible limiting causes of dog vaccination efficiency may be an insufficient coverage of the dog population due to the inaccessibility of unrestricted or aggressive dogs to parenteral vaccination. To address this problem, several authors investigated the possibility of using oral vaccination as a complementary measure. Three areas of research were explored using the SAG2 vaccine, a highly attenuated double mutant of the attenuated SAD Bern rabies strain [16]: a) the possibility of conferring immunity to the dogs by the oral route [11, 19]; b) the innocuity of this candidate vaccine for target and local non-target species [11, 12, 14, 20, 21]; and c) the possibility of increasing the vaccination coverage of the dog population through baits and bait distribution systems [13, 15, 17, 18].

It has already been shown that SAG2, currently used with success in France and Switzerland for the control of fox rabies [2], is also an effective vaccine for oral immunisation of dogs. This has been demonstrated in laboratory beagles that received either the liquid vaccine orally on the tongue or in baits [11]. Considering the high temperatures that generally prevail in the areas where dog rabies is endemic, a freeze-dried bait called DBL2 has been developed to ensure a high stability of the SAG2 vaccine titre. This bait has been tested on laboratory beagles and has conferred on them protection against a subsequent rabies challenge [19]. Following parenteral vaccination, however, native Tunisian dogs generally produce lower antibody titres than dogs owned in Europe, which themselves produce lower titres than laboratory dogs [6]. Because the same problem could occur when oral vaccination is applied to stray dogs, our objective in the present study was to verify that local Tunisian dogs were efficiently protected against rabies when vaccinated with a single SAG2 vaccine DBL2 bait whose efficacy has already been demonstrated in laboratory dogs.

2. MATERIALS AND METHODS

2.1. Animals

Fourteen dogs of both sexes and of various ages were obtained from 12 owners in Tunisia. All belonged to the undifferentiated local mongrel breed. According to the testimony of their owners, all adult dogs (six individuals older than 1 year) and young dogs (three individuals less than 1 year and more than 3 months old) had never been vaccinated against rabies. The five

puppies (less than 3 months old) were born from three non-vaccinated bitches. Dogs were housed in individual cages in the animal facility of the Institut de la recherche vétérinaire de Tunisie, Tunisia (IRVT), watered at will and fed every day with commercial dry dog food. They were vaccinated against distemper, leptospirosis, hepatitis and parvovirus (Tetradog[®], Merial, Lyon, France). In order to keep the dogs in conditions similar to the ones that prevail locally, they were not given any treatment against the several parasitic diseases with which they were affected (*table I*). At the beginning, they were randomly divided into two groups. When baits were presented to the dogs, however, four puppies were unable to break the bait and for this reason we swapped them with older individuals of the control group (*table I*).

2.2. Vaccine

SAG2 is a live modified rabies virus vaccine. This strain originated from a virus isolated from the salivary glands of a rabid dog in 1935, subsequently passaged on to mice, chick embryos, then various cell lines [7]. Following the protocol described by Seif et al. [22], the SAD Bern virus was incubated with ascites fluid contain-

ing the 50AD1 monoclonal antibody. The mutants escaping neutralisation were isolated by plaque purification on CER cells. Their patterns of sensitivity to monoclonal antibodies were determined and compared to those of the parent SAD Bern strain. The mutation of the genome encoding amino acid 333 of the glycoprotein was determined by dideoxynucleotide sequencing. The pathogenicity of emerging clones was checked by intra-cerebral inoculation in adult mice [9, 16]. The resulting SK clone was again subjected to all of the above-mentioned treatments, except that the 50AC1 antibody was used. Thus, the SAG2 vaccine strain, which is characterised by a double mutation in position 333, was selected, therefore suggesting a greater genetic stability of the attenuating mutation.

The lyophilised SAG2 vaccine was presented in a DBL2 dry dog bait [19] developed by the Virbac Laboratories (Carros, France). Briefly, the SAG2 suspension was formulated within an excipient specifically designed for freeze-drying (patent application) and positioned into thermoformed moulds before undergoing a freeze-drying cycle. The resulting cubes were coated with a palatable matrix similar to that used for the Rabigen® SAG2 oral fox bait. The DBL2 baits were packed in plastic bags and stored at 4 °C until use.

Table I. Distribution of dogs in the experimental groups and summary of the clinical examination.

Group	Dog	Sex	Age group	General appreciation status of health	Other observations
I: vaccinated	1	F	puppy	medium	ticks
	2	M	adult	good	–
	3	M	adult	medium	ticks
	4	M	adult	good	fleas
	5	F	adult	medium	fleas, conjunctivitis
	6	F	young	medium	conjunctivitis
	7	F	adult	medium	hair loss
II: control	8	F	puppy	good	–
	9	F	puppy	good	ticks
	10	F	puppy	medium	ticks
	11	F	young	medium	fleas
	12	M	young	medium	ticks, light anaemia
	13	M	puppy	medium	ticks, light anaemia
	14	M	adult	apparently good	ticks, fleas, scar on face, respiratory noise, cardiac arrhythmia

The DBL2 baits were transported from the Virbac Laboratories to the IRVT on dry ice by special delivery and kept at +4 °C until use, 11 days later. On day 0 (7 May 1996), seven baits were weighed, and each bait was presented to each dog of group I for free consumption. Dogs had been fasting for 24 h and were not sedated. No food or water was given to them simultaneously with the bait. They were observed for 15 min, and subsequently the remains of the baits, if any, were carefully collected and weighed. The titre, determined on the remaining baits carried back to the Virbac Laboratories, was $10^{8.3}$ TCID₅₀ per bait.

2.3. Challenge

All dogs (groups I and II) were inoculated in the temporal muscle with 1 mL of a rabies virus suspension of the Tunisian canine street rabies virus 33 days after distribution of the baits to the seven dogs in the vaccination group (group I). The titration of the inoculum in mice demonstrated that the dose inoculated in each dog was $\leq 10^4$ mouse intra-cerebral LD₅₀.

2.4. Observation, serum sampling and post-mortem examination

All dogs were observed daily until death or euthanasia and carefully examined for any symptom suggestive of rabies. Euthanasia was performed on day 160 post-challenge with an intravenous injection of Dolethal® (Vétoquinol, Lure, France).

Blood samples were collected from all dogs on day 0 to verify that they were void of the rabies antigen, and on days 7, 14, 21, 33 and 40 (= 7 days after challenge), as well as on day 193 (= day of euthanasia). Neutralising antibodies specific for the rabies virus were determined using the fluorescent antibody virus neutralisation (FAVN) test [3]. According to previous results obtained with this test, the adopted threshold for positivity was 0.24 IU/mL [8].

After death, brain tissue (Ammon's horn, cortex and bulb) was tested by the fluorescent antibody technique (FA) [10] and negative samples were additionally inoculated into the N2a cell culture [4].

2.5. Statistical analysis

The survival rates between groups were compared using the Fisher exact test [23].

3. RESULTS

All seven dogs were apparently interested by the bait. Five dogs smelled it and consequently licked it. They all chewed the bait. Six of the dogs consumed the whole bait: within less than 1 min (two dogs), within 1 min (two dogs), 3 min (one dog) or 6 min (one dog). Dog 1 consumed only 5 g of the bait during 5 min and did not pay any attention to the remaining bait for the rest of the observation period.

As detailed in *table II*, no dog had a significant rabies antibody titre at day 0. Similarly, until the challenge, the dogs of the control group (group II) did not have significant titres, with the exception of dog 14 that showed significant titres from day 14 onwards (even though it had not received a bait). Only two dogs (dogs 5 and 7) out of the seven of group I developed a significant antibody titre as a response to bait uptake and before the challenge. This significant titre was detected from the 14th day after the bait uptake onwards. On the 7th day after the challenge, control dog 14 still had the only significant titre observed in group II. In group I, the two dogs that developed antibodies after vaccination had increased titres and two more dogs (dogs 1 and 4) also produced significant titres. On day 160 after the challenge, these four dogs out of the five surviving dogs of group I still had significant neutralising antibody titres.

On days 19 and 22 after the challenge, two dogs of group I displayed abnormal behaviour with successive spells of apathy or agitation. After they remained recumbent, they were found dead 3 and 5 days later. On days 12, 13 (two dogs), 18 and 92, five dogs of group II showed similar symptoms and were found dead 2–4 days later. The FA test was strongly positive for each of

Table II. Neutralising rabies antibody titre (IU per mL) in dogs determined by the fluorescent antibody virus neutralisation (FAVN) test (significant titres are in bold characters).

Group	Dog	D ₀	D ₇	D ₁₄	D ₂₁	D ₃₃ Challenge	D ₄₀	D ₁₉₃
I: vaccinated at D0	1	0.14	0.06	0.06	0.03	0.06	5.00	0.72
	2	0.06	0.04	0.04	0.04	0.06	0.06	dead
	3	ND	0.06	0.08	0.05	0.06	<0.04	dead
	4	0.06	0.03	0.06	0.03	0.02	0.95	0.32
	5	0.06	0.06	5	5	2.88	5.00	5
	6	<0.04	<0.14	0.08	0.05	0.08	0.06	0.04
	7	0.08	0.06	0.72	0.95	0.32	2.88	0.95
II: control	8	0.04	0.05	0.05	0.03	<0.02	0.04	dead
	9	<0.06	0.04	0.04	0.05	0.03	0.04	dead
	10	0.14	0.03	0.05	0.04	0.14	0.08	dead
	11	0.06	0.08	0.05	0.03	0.08	0.06	0.04
	12	0.11	<0.18	0.06	0.05	0.06	0.04	dead
	13	0.08	0.06	0.02	0.02	0.06	<0.02	dead
	14	0.06	<0.18	1.66	0.72	3.79	5	1.26

* ND = not determined.

these seven dogs, thus confirming the rabies etiology. For all euthanised dogs (five dogs of group I and the only surviving dog of group II), the FA test was negative and this result was confirmed by cell culture. When excluding dog 14 from the control group considering that it had specific rabies antibodies before challenge, the survival rate of vaccinated animals (5/7) is higher than the observed rate in controls (2/7) at an unilateral risk of 7.8 % according to the Fischer exact test.

4. DISCUSSION

Many difficulties are encountered when experiments must be conducted using dogs of varying and generally poor health obtained from the field. Conditions are not standardised and uncertain health status introduces an uncontrolled variability into the experimental results. As described in *table I*, the dogs were not uniformly parasitised. To reflect real conditions, we did

not treat the dogs against parasites. In contrast with the conditions commonly prevailing in Tunisian stray dogs, however, they were nutritiously fed. In order to avoid the 100 % mortality of dogs that we had observed in a previous attempt to initiate this trial, we had to vaccinate the dogs against the most threatening diseases other than rabies. This treatment could have improved the ability of these dogs to respond to the oral vaccination compared to dogs vaccinated in the field. The time period spent at the IRVT before vaccination was, however, very short (only 2–32 days) and did not allow a real improvement in the health status currently observed in street dogs. Additionally, considering that an interaction between the vaccines (Tetradog and SAG2) could not be excluded when administered within a short period, vaccination with SAG2 took place 23 days later for all dogs of group I (except for dog 5 for which the interval was 34 days).

The fact that several vaccinated dogs did not show a significant seroconversion and

nevertheless survived the subsequent challenge has already been described in other rabies vaccination experiments (review in Aubert [1]). This phenomenon seems more common when vaccination is performed orally and has also been observed after oral vaccination of dogs with the SAG2. According to Fekadu et al. [11], two dogs out of ten laboratory dogs vaccinated with a bait containing $10^{7.5}$ suckling mice intra-cerebral LD_{50} (SMICLD₅₀) did not seroconvert before challenge, while all of the ten dogs vaccinated with $10^{8.5}$ SMICLD₅₀ seroconverted. In the first experiment conducted with the freeze-dried DBL2 bait, none of the eight laboratory dogs produced antibodies [19].

Three vaccinated animals did not show significant humoral response 7 days after challenge and two of them died of rabies. We interpreted this failure as an absence of efficient contact of the lyophilised vaccine with the oropharyngeal mucosa. Interestingly, one of these dogs, dog 1, which consumed only one fourth of the bait, survived. Also, the survival of dogs cannot be explained by the time they took to consume their bait. We interpret the high titre shown 7 days after challenge by dogs 1, 4, 5 and 7 as an anamnestic response to the antigenic stimulation given by the challenge. Among the controls, only dog 14 developed a high titre at this time.

To interpret the results we had to exclude dog 14 from the analysis: the significant antibody titre in this animal before challenge and its anamnestic response after challenge suggested that this dog had previously been in contact with the rabies antigen either through a non-lethal infection (review in Aubert [1]) or, more likely, through vaccination despite the declaration of the dog owner. Therefore, we conclude that the partial or total consumption of a single DBL2 bait protected five out of seven indigenous Tunisian dogs against a challenge with a local canine street rabies virus strain that killed five out of six non-vaccinated dogs. The survival rate of vaccinated animals is

higher than the observed rate in controls with a unilateral risk of 7.8 %. While the statistical risk is generally considered as significant when it is less than 5 %, these results obtained in Tunisian dogs nevertheless confirmed the results already obtained with the DBL2 bait on laboratory dogs: three out of four dogs vaccinated with one DBL2 bait containing 10^8 TCID₅₀ were protected against a challenge of more than one dog-LD₁₀₀ of a canine street rabies strain (MA 85 strain) and we obtained the same result for four other dogs vaccinated with one DBL2 bait containing 10^9 TCID₅₀ [19]. In conclusion, we observed that the survival rate of dogs that consumed one DBL2 bait at least partially was 6/8 and 5/7 in laboratory dogs and indigenous Tunisian dogs, respectively, and it was not significantly different between both groups.

These findings provide clear evidence that the DBL2 bait, carrying the SAG2 vaccine in a lyophilised form is a good candidate for vaccinating stray dogs by the oral route.

Following the demonstration of the efficacy of the SAG2/DBL2 vaccine presentation in laboratory and indigenous dogs as well as the safety in the most common north African non-target species [14], the next step is to investigate its performance under field conditions, possibly in comparison with the liquid-filled bait used to vaccinate foxes in Europe.

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