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## ABSTRACT

Parenteral vaccination campaigns are integral to the elimination of canine rabies. To maximize herd immunity in dogs, immunocontraception provided at the time of rabies vaccination should reduce fecundity and dog abundance. GonaCon™ has been used successfully as an immunocontraceptive in a variety of mammals, and by inference, the dog would be an ideal candidate for testing. As an initial step in evaluating a combination-vaccination program, we assessed the effects of GonaCon™ on rabies virus neutralizing antibody production in dogs after administration of a veterinary rabies vaccine. Eighteen feral/free ranging dogs were included in this initial study: six were given GonaCon™ only, six were given rabies vaccination only, and six received GonaCon™ and rabies vaccination. Antibody levels were evaluated over 82 days. The use of the immunocontraceptive GonaCon™ did not affect the ability of dogs to seroconvert in response to the rabies vaccine. Thus, GonaCon™ provides a potential immunocontraceptive for use in combination with rabies vaccine to increase herd immunity and address dog population over abundance to better manage rabies.

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## 1. Introduction

During the last century, extensive parenteral vaccination of host reservoir dog populations accompanied by control of free ranging dogs has led to the elimination of canine rabies in several countries, such as Japan [1] and the United States [2]. Almost a half of a century later, rabies continues to challenge public health systems in developing countries, especially in Africa and Asia, where many of the estimated 55,000 annual human rabies deaths occur. Rabies is a neglected tropical disease [3,4] due in part to a lack of political will brought about by an absence of accurate data on the true public health impact of this zoonosis [5].

The threat of rabies virus transmission to humans from dogs increases when the density of dogs exceeds the threshold density at which canine rabies is maintained. The threshold density for canine rabies has been estimated to be 4.5 dogs/km<sup>2</sup> [5]. Immunocontraception has been proposed to reduce the density dependence of rabies [6]. Several targets for immunocontraception are avail-

able, such as gonadotropin-releasing hormone (GnRH). Research has suggested that by controlling GnRH, the breeding capability of a dog could be removed [7]. Others have shown that a GnRH vaccine reduced the breeding capability of male dogs for at least 1 year [8]. In other species tested, GonaCon™ induced infertility 2–3 times longer in females than males. Thus, we expect the same increased induced infertility in female dogs.

The potential for interference between simultaneously administered immunocontraceptive and rabies vaccines must be determined before field trials using this tandem vaccination strategy merit consideration. In this study, we evaluated the effects of the immunocontraceptive vaccine GonaCon™ on rabies virus neutralizing antibody (VNA) induction in dogs from a commercial rabies vaccine.

## 2. Materials and methods

### 2.1. GonaCon™ and rabies vaccine

The GonaCon™ immunocontraceptive vaccine (United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, National Wildlife Research Center, Fort

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Collins, CO, USA) formulation consisted of mammalian GnRH conjugated to a large mollusk hemocyanin protein (keyhole limpet hemocyanin [KLH]) and emulsified with the adjuvant in Dog-AdjuVac™. Each 0.5-ml dose contained 500 µg of the GnRH conjugate and 85 µg (one-half the standard dose) of inactivated *Mycobacterium avium*. GonaCon™ was stored and shipped under refrigerated conditions in pre-loaded, 3 ml Air-Tite luer-hub syringes (Air-Tite Products, Virginia Beach, VA, USA).

The rabies virus vaccine was a commercially available inactivated rabies virus (DEFENSOR 3, Pfizer, Inc., New York, NY, USA). Each 1 ml container was stored under refrigerated conditions at 4 °C until ready for use.

## 2.2. Experimental animals

Eighteen female dogs of mixed breeds were acquired from dog round-ups conducted by the Navajo Nation Animal Control Program during May 2008. Unclaimed dogs gathered by Animal Control are euthanized 3 days post round-up pursuant to the Navajo Nation Animal Control Laws (Navajo Tribal Code; Title 13, Section 1711, Impounded Animals). Female dogs over 5 months of age were offered for this study instead of euthanasia. Breeding age female dogs were selected based on availability.

Dogs were separated into kennels at the Navajo Nation Animal Control Program's Many Farms Facility, Many Farms, Arizona, USA. Each dog was individually marked with a collar and an attached identification tag. Animal care personnel maintained the kennels twice per day. Dogs were fed on commercial dog food (Ol' Roy: Complete Nutrition Dog Food, Wal-Mart Stores, Inc., Bentonville, AR, USA) and offered water *ad libitum*. All dogs were under direct oversight by an onsite veterinarian. Dogs were randomly assigned to each of three treatment groups.

**Group 1:** Six animals. All were vaccinated with 0.5 ml of GonaCon™ immunocontraceptive. The injection was administered intramuscularly in the upper left hind leg.

**Group 2:** Six animals. All were vaccinated with a single 1 ml injection of DEFENSOR-3 rabies vaccine. The vaccine was administered intramuscularly to the upper right hind leg.

**Group 3:** Six animals. Each animal received a single injection of 0.5 ml GonaCon™ immunocontraceptive plus a 1 ml injection of DEFENSOR-3 rabies vaccine. The immunocontraceptive injection was administered intramuscularly to the upper left hind leg and the rabies vaccine was administered intramuscularly to the upper right hind leg.

## 2.3. Determination of anti-GnRH antibody titers

Blood was drawn from the jugular vein of each dog on a weekly basis using a 12 ml syringe equipped with a 20-gauge needle, or with a 6 ml CorVac™ (Kendall, Mansfield, MA, USA) serum separator tube and a 20-gauge needle. Blood samples were stored on blue ice in an ice chest and then centrifuged. Serum was separated and stored in CryoTubes™ (Nalge Nunc International, Rochester, NY, USA) at -70 °C until analyzed.

To evaluate the immune responses of dogs to vaccination with GonaCon™, we used an enzyme-linked immunosorbent assay (ELISA) to measure anti-GnRH antibody titers [8]. In brief, a 96-well plate was prepared by adding 100 ng of BSA-GnRH antigen to each well and then blocking with SeaBlock™. Fifty microlitres of serially diluted serum was used for each assay. Anti-GnRH antibody was determined by adding rabbit anti-dog IgG to each well, washing, then adding goat anti-rabbit IgG labeled with horse-radish peroxidase (HRP), washing, then developing a color by adding a HRP substrate. The color was proportional to the anti-GnRH anti-

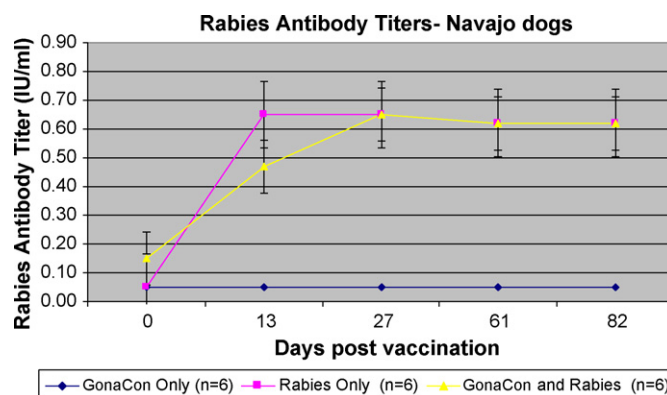


Fig. 1. Mean rabies virus neutralizing antibody titers in dogs vaccinated with DEFENSOR-3 rabies vaccine and GonaCon™.

body titer. BSA-GnRH was added to the ELISA plate, causing only antibodies to GnRH to be detected.

## 2.4. Determination of rabies VNA titers

The coded sera were thawed rapidly and heat-inactivated in a 56 °C water bath for 1 h. Rabies VNA titers were determined using the Rapid Fluorescent Focus Inhibition Test (RFFIT), as previously described [9]. In brief, the RFFIT was performed by mixing dilutions of test serum with a constant amount of challenge virus standard (CVS) rabies virus, 50 FFD<sub>50</sub>/0.1 ml, in multi-channeled slides (LabTek, Thermo Fisher Scientific, Rochester, NY). After allowing the mixture to incubate in a CO<sub>2</sub> incubator at 37 °C for 90 min, mouse neuroblastoma (MNA, C1300) cells in Eagle's Minimum Essential Medium with 10% (heat-inactivated) fetal bovine serum (MEM-10) were added to each serum-virus mixture. The serum-virus-cell cultures were incubated for 20 h in a CO<sub>2</sub> incubator at 37 °C. Thereafter, the cultures were taken from the incubator, the media was removed, and the slides were washed with PBS, fixed in acetone, and stained with an anti-rabies virus conjugate. The fixed cell cultures were observed under a fluorescence microscope for the presence of fluorescing cells; 20 microscopic fields (160×–200×) were read for each dilution and compared against a reference slide (virus control; 50 FFD<sub>50</sub>/0.1 ml), which contained 18–20 fields of undiluted fluorescing cells. The 50% neutralization endpoint was defined as the dilution at which 9 or less of the 20 observed fields contained one or more infected cells. Observed titers were converted into international units by comparison with the standard positive control rabies virus immunoglobulin (IgG) at a concentration of 2 IU/ml.

## 2.5. Statistical methods

SAS software was used for statistical analysis [10]. The GLM procedure for repeated measures of analysis of variance and Tukey's studentized range were used for comparison. Statistical differences with  $P < 0.05$  were considered significant.

## 3. Results

The anti-GnRH antibody and rabies VNA titers for each group were determined over an 82-day period (Fig. 1). Titers were analyzed at days 0, 13, 27, 61, and 82 as shown in Table 1. Group 1 was an internal control for the rabies vaccinations administered to Groups 2 and 3. In Group 3, for those individuals that received both vaccines, antibody responses to GnRH and rabies virus were measured (Fig. 2).

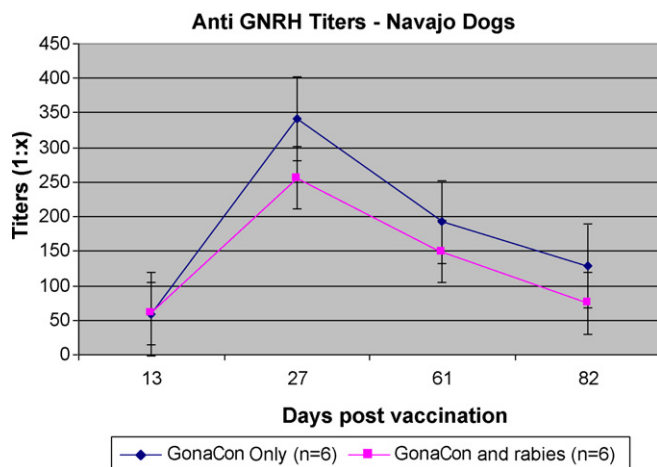
**Table 1**  
Tukey's studentized range (HSD) test.

Treatment comparison	Difference between means	Simultaneous 95%
Day 0		
G3–G1	0.12000	-0.10731, 0.34731
G3–G2	0.12000	-0.10731, 0.34731
G1–G3	-0.12000	-0.34731, 0.10731
G1–G2	0.00000	-0.21673, 0.21673
G2–G3	-0.12000	-0.34731, 0.10731
G2–G1	0.00000	-0.21673, 0.21673
Day 13		
G2–G3	0.10800	-0.09658, 0.31258
G2–G1	0.60000	-0.40494, 0.79506***
G3–G2	-0.10800	-0.31258, 0.09658
G3–G1	0.49200	0.28742, 0.69658***
G1–G2	-0.60000	-0.79506, 0.40494***
G1–G3	-0.49200	-0.69658, -0.28742
Day 27		
G2–G3	0.0000	0.0000, 0.0000
G2–G1	0.6000	0.6000, 0.6000***
G3–G2	0.0000	0.0000, 0.0000
G3–G1	0.6000	0.6000, 0.6000***
G1–G2	-0.6000	-0.6000, -0.6000***
G1–G3	-0.6000	-0.6000, -0.6000***
Day 61		
G2–G3	0.0000	0.0000, 0.0000
G2–G1	0.5700	0.5700, 0.5700***
G3–G2	0.0000	0.0000, 0.0000
G3–G1	0.5700	0.5700, 0.5700***
G1–G2	-0.5700	-0.5700, -0.5700***
G1–G3	-0.5700	-0.5700, -0.5700***
Day 82		
G2–G3	0.0000	0.0000, 0.0000
G2–G1	0.5700	0.5700, 0.5700***
G3–G2	0.0000	0.0000, 0.0000
G3–G1	0.5700	0.5700, 0.5700***
G1–G2	-0.5700	-0.5700, -0.5700***
G1–G3	-0.5700	-0.5700, -0.5700***

\*\*\* Comparisons significant at the 0.05 level.

There was a significant interaction between time step ( $F = 56.59$ ,  $P < 0.0001$ ) and group ( $F = 16.28$ ,  $P < 0.0001$ ). This interaction was a function of the negative rabies VNA titers for all individuals at time-step-day 0 and the subsequent rise in titers for Groups 2 and 3 post time-step-day 0.

At time-step-day 0, there was no difference in mean rabies titers by group. For time-steps 1–4 (days 13, 27, 61, and 82) there were statistically significant differences for the same treatment



**Fig. 2.** Post GnRH antibody titers following GonaCon™ and rabies vaccination in dogs.

group comparisons (between Group 3, Group 1 and Group 2). For time-steps 1–4 (days 13, 27, 61, and 82), there was no statistically significant difference (i.e., no evidence of interference) in mean rabies VNA titers for the Group 2 (rabies only) and the Group 3 (rabies + GonaCon™) comparison.

#### 4. Discussion

To our knowledge, this is the first use of the immunocontraceptive GonaCon™ in female dogs. We have demonstrated the potential to use this immunocontraceptive in breeding age female dogs without affecting parenteral rabies immunization in 100% of vaccinated animals.

Throughout the world, there is a critical need to control population growth in dogs, especially in areas where canine rabies remains endemic. The current practice of euthanizing large numbers of dogs is a social concern [11], plus it is labor intensive limiting its potential across broad geographic areas. Alternatives to euthanasia have focused on chemical and surgical sterilization [12,13], each possessing positive and negative attributes. Currently applied dog population control methods have been unable to affect a sufficient sized cohort of dogs within a reasonable time frame to have an impact on the local population.

More effective rabies control measures in dogs may be possible through injectable immunocontraceptive products administered in tandem with rabies vaccination programs. Ultimately, the factors that would influence the population-level response to immunocontraception are the proportion of females that can be treated, the incidence of non-responders among treated dogs, the fertility of untreated dogs, compensatory responses such as increased reproductive rates in untreated animals and increased survival of treated individuals, including rates of mortality, emigration and immigration. Given that no interference was identified between GonaCon™ and a commercial rabies vaccine in this immunogenicity trial, future research should determine the potential effects of immunocontraception upon duration of immunity and efficacy against relevant challenge viruses.

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