

PRESENTATION SUMMARY & POWERPOINT

Potential of Canine Zona Pellucida Glycoproteins-Based Immunocontraceptive Vaccines

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Feline and canine eggs are enveloped by an acellular translucent matrix termed as zona pellucida (ZP). The ZP matrix plays a critical role in reproduction by acting as a docking site for relatively species-specific recognition and sperm binding to the oocyte, leading to successful fertilization and also protection of the embryo during early stages of development, prior to implantation. Biochemical analysis of the ZP matrix revealed that it is primarily composed of 3-5 glycoproteins depending upon the species studied. A great deal of information on their structure and function during the complex process of fertilization is available from several species (Wassarman, 1999; Spargo and Hope, 2003; Gupta et al., 2006). As zona proteins play a pivotal role during fertilization, these have been proposed as candidate antigens for development of a contraceptive vaccine. The basic principle of such a vaccine is to generate specific and adequate antibody response, which thereby interferes in sperm-egg interaction leading to inhibition in fertility. Initially, these experiments were carried out using ZP glycoproteins isolated from porcine oocytes due to their easy accessibility from abattoirs (Kirkpatrick et al., 1996; Gupta et al., 2004). Further, the antibodies generated against porcine ZP glycoproteins showed immunological cross-reactivity with ZP from a wide variety of animal species. This has allowed heterologous immunization as an approach for development of contraceptive vaccines. However, porcine ZP has not shown very promising results with respect to their contraceptive efficacy in cats (Jewgenow et al., 2000).

In order to develop a contraceptive vaccine based on ZP glycoproteins for controlling the population of street dogs, we have cloned and expressed canine zona pellucida 2 (ZP2) and zona pellucida 3 (ZP3) in *E. coli*. The process for their large-scale production and subsequent purification was optimized (Santhanam et al., 1998; Srivastava et al., 2002). Analysis by SDS-PAGE as well as Western blot of the purified recombinant dog ZP2 revealed a major band of 70 kDa. Recombinant dog ZP3 showed a major band of 42 kDa and an additional band of 32 kDa. The purified recombinant proteins were conjugated with diphtheria toxoid (DT) used as "carrier" protein to generate antibody against "self" protein. Nondescript female dogs were immunized intramuscularly in the hind limbs at two sites with ZP2-DT and ZP3-DT conjugates employing permissible adjuvants such as Squalene and Arlacel-A. A third group of female dogs was immunized with DT as a control group. Sodium phthalyl derivative of lipopolysaccharide (SPLPS) was also included in the first injection as an additional adjuvant. Primary immunization of dogs comprised three injections of the respective proteins at monthly intervals. High antibody titers were generated against dog ZP2 and ZP3 proteins as well as the carrier, when immunized with the respective recombinant protein-DT conjugates (Srivastava et al.,

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2002). However, curtailment of fertility was observed only in the ZP3-DT immunized group of female dogs. Immunization of female dogs with either DT or ZP2-DT conjugate failed to block fertility and these animals conceived when mated during the period of heat with males of proven fertility. Histopathology of ovaries revealed that the block in fertility observed in the group of female dogs immunized with recombinant ZP3 conjugated to DT is probably manifested by follicular atresia and degenerative changes in the oocytes.

In several countries, including India, the increasing population of street dogs is a significant problem. These dogs are not owned by anyone. It has led to high incidence of rabies, which is a major zoonotic disease of significant public-health concern in many parts of the world, especially in developing countries, where it is endemic among dogs. Keeping in view the encouraging results with bacterially expressed recombinant dog ZP3, canine ZP3 was also expressed in baculovirus expression system with the notion that the glycosylated form of the protein may be more immunogenic and thus confer improved contraceptive potential. Ni-NTA purified recombinant dog ZP3 revealed a band of ~50 kDa by SDS-PAGE as well as Western blot analysis. As an alternate to chemical conjugation of the recombinant dog ZP3 with DT for active immunization studies, it was expressed as a fusion protein along with rabies glycoprotein-G (RV-G, an antigen capable of generating antibodies that can confer protection against rabies infection) in baculovirus expression system. Such a vaccine construct, if successful, will not only inhibit fertility in dogs but may also provide protection against rabies infection. Active immunization studies carried out in nondescript female dogs with the recombinant dog ZP3-RV-G resulted in generation of antibodies against both ZP3 and RV-G as determined by ELISA. In this group of immunized animals, 4 out of 6 animals came to heat. Out of 4 animals, 2 failed to conceive and the other 2 animals became pregnant. Immunization of female dogs with a physical mixture of recombinant dog ZP3-DT conjugate and recombinant RV-G showed promise of contraception. Only 2 immunized female dogs came into heat out of the 4 immunized animals and both failed to conceive when mated with males of proven fertility. These experiments suggest that dog ZP3 can be a promising candidate for development of a contraceptive vaccine for controlling the street dog population and encourage us to carry on large-scale efficacy trials.

To make a contraceptive vaccine based on canine ZP3 as practical and user-friendly as possible, convenient vaccine delivery systems need to be developed, with an aim toward minimizing the number of injections, preferably single inoculum. Alternate routes of immunization, such as oral delivery, should also be explored. To achieve the above, we have initiated work in our laboratory to entrap canine ZP3 in polylactide microspheres with an aim toward giving a single injection that might suffice to produce adequate antibody response for 6-9 months. Attempts are also going on to obtain recombinant dog ZP3 incorporating "promiscuous" T non-B cell epitopes. This approach will avoid chemical coupling of ZP3 with DT. Immunized animals will be devoid of high circulating antibodies against the "carrier" protein and thereby may also circumvent "carrier-mediated" suppression in the immune response sometimes observed (Sad et al., 1991). Progress in this direction will be presented. In addition, the ability of a canine ZP3-based

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DNA vaccine to generate immune response in mice (Rath et al., 2003) will also be discussed.

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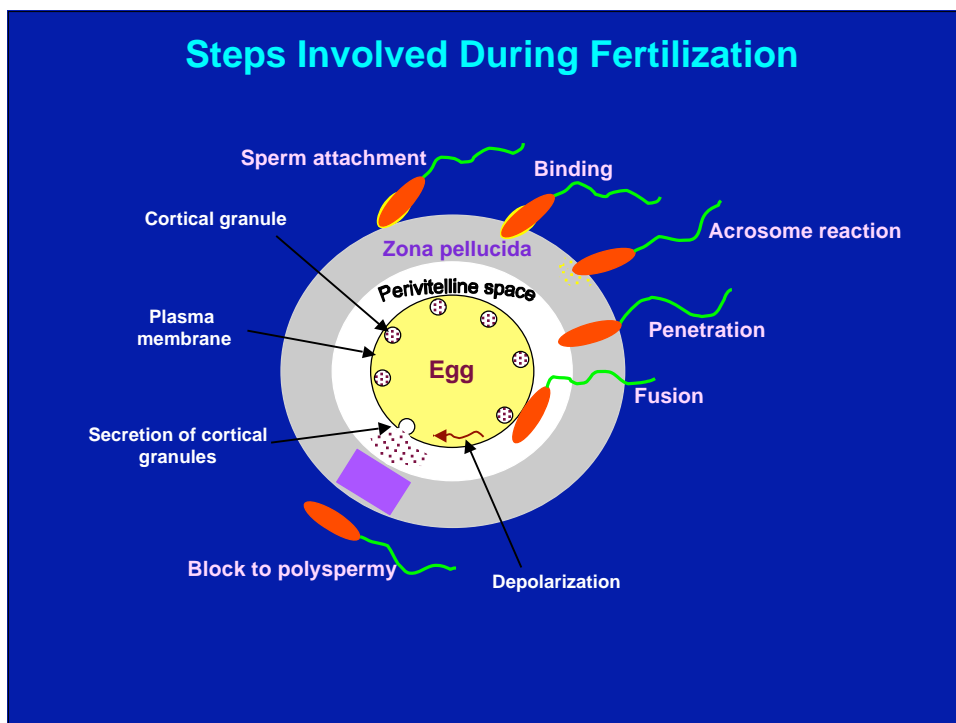
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By Dr. S.K. Gupta

POTENTIAL OF CANINE ZONA PELLUCIDA GLYCOPROTEINS BASED IMMUNOCONTRACEPTIVE VACCINES



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FERTILITY REGULATING VACCINES

Zona Pellucida as Target Antigens

- Critical role in fertilization
- Tissue and stage specific expression
- Demonstrated effect of antibodies to block fertility
- Active immunization lead to variable degree of ovarian dysfunction

Probable factors for the ovarian dysfunction

- Contamination by other ovarian associated proteins
- Adjuvants
- Oophoritogenic T cell epitopes
- Susceptibility of the animal model

Native source

- a) Availability in small quantities
- b) Presence of contaminating ovarian proteins – ovarian pathology

Expression in heterologous expression system

Active immunization of female baboons and bonnet monkeys with *E. coli*-expressed recombinant monkey ZP2 and/or ZPB (ZP4) conjugated with DT blocks fertility (Govind and Gupta (2000) Vaccine 18: 2970; Govind et al (2002) Vaccine 21: 78)

SHORTCOMINGS OF IMMUNOCONTRACEPTIVE VACCINES FOR HUMAN USE

- Failure to Generate Adequate Protective Antibody Response in 100% of the Recipients
- Variation in the Duration of Protective Antibody Response among the Immunized Individuals

NONETHELESS, IMMUNOCONTRACEPTIVE VACCINE IS AN ATTRACTIVE PROPOSITION FOR WILDLIFE POPULATION CONTROL

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☒ Stray dogs

An ancient canine breed known as the **Pariah**, which has existed all over Asia and North Africa ever since human beings started living in settlements. They are, and have always been, scavengers – that is, they live on garbage created by humans. **Mongrel** or mixed breed adds to stray dog population. **There are approx. 0.2 to 0.4 million stray dogs in Delhi alone.** Two dogs can multiply to over 300 in 3 years.



☒ Problems caused by stray dogs

- Dog bites
- Rabies – a fatal disease which can be transmitted to humans (in late 1980s and early 1990s, 50 human deaths due to rabies in Mumbai; 215 cases of rabies death reported in Delhi in the year 2004; incidence of rabies mediated death is increasing)
- Barking and howling

☒ Population control measures

- Catching and slaughtering not very effective in long run (Ethical considerations)
- Mass sterilisation program of stray dogs by non-government organization; under general anaesthesia; neutering; females-ovaries and uterus removed; males-testicles removed

Is an immunocontraceptive vaccine feasible that can be delivered along with rabies vaccine?

AMINO ACID SEQUENCE OF DOG ZP2

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1  IACRGGDGGGSDP%SKF%SAIDNSTYRSLSLFFLLFFSVRSVGVRLVHPLEP 50
51  GTVICHERRSTVEEPRRLGTRKVBHGVVDPESEELLNCTSLDPERLTLK 100
101  APSEFCRIRVLAGHRLALDLDHRAASHURAPRYQLSCFVHQTEETDERRA 150
151  GELCTKDEKSEFTERRLFGADENTHRSKGRMBRVDVDRKAGMLTLDEAL 200
201  HQGMRLEPDSHRELSVAVSPHATEVTHVQENSHLVTVPLKLEHESFGOK 250
251  ILTTRVLPDSDPFTFNATHMLTELEFGEKLRVRENTMFRVTVLIRHRS 300
301  LDKKELRGLRLHFDANLKRHFERKCLLYDFMLALRLTEAFERDFVTV 350
351  VQECVCEPVPVLEVFGMLCFADGERDVFVSHRFRRLHLDLRYVGDSSC 400
401  QPFRKAPFQGLTEPHIPLRGGCTHLEKQDVTVVEHEIHALVFDLPPSTI 450
451  SRDSEPRRTVKCHVSRDDELDTDFVQSLPPVAVSVRPGRLMLTLOTVPD 500
501  KSLRFPXGRRKXPVVRCLRQTYLEVKVLRRAOPHRLVLDCCUATPTI 550
551  DPARHPQVRLVDRGCVRLDDBYRTTFHPVGSVLYPTRYQRDVKIEAFEL 600
601  SEAGVLSLVVCHTALCHRLSPDPLFCVFCVVSRRHRRATGSTEERK 650
651  HRTVSLPFPFLLADSSRDGVDSRGRAGVVAPEVTVVAVKALRGLVVA 700
701  LGLTVLDEKDTMVLRI 746
    
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AMINO ACID SEQUENCE OF DOG ZP3

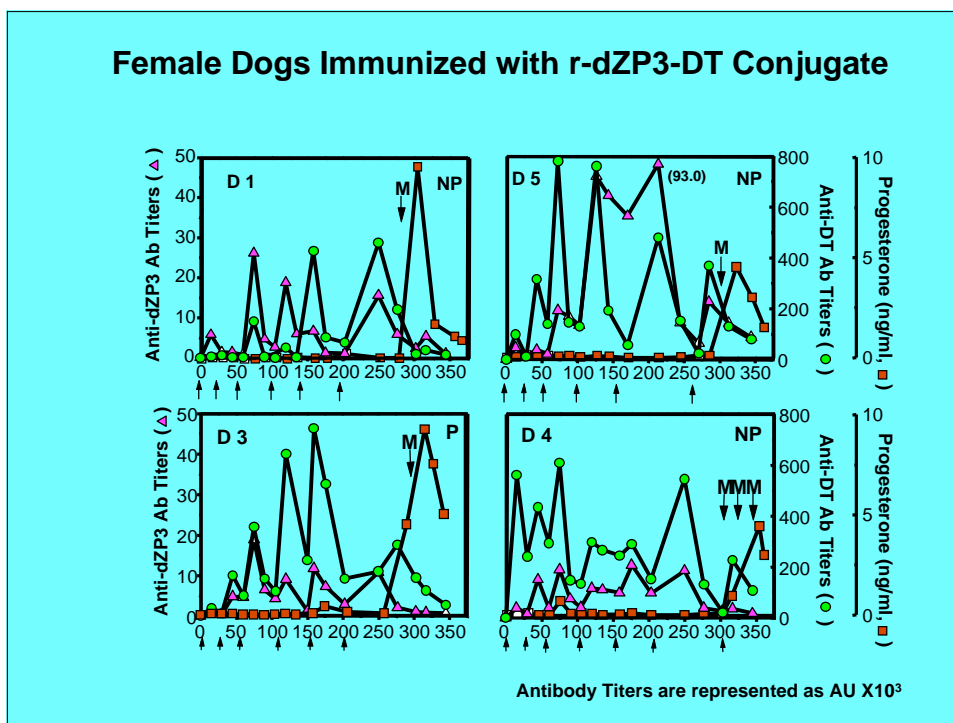
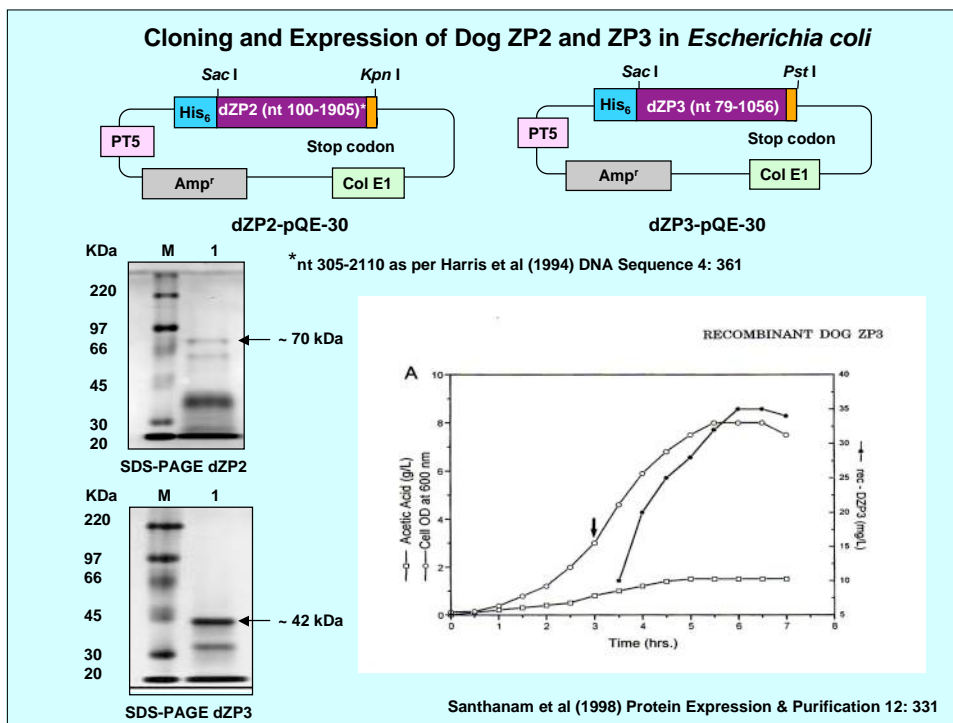
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1  NGLSKGFICFLLLGGRELCCNFIIMPTEYVPLTSRPPVNVDCLESQLV 50
51  VIVSKDLFCFKLIRPADELGPNCEPLVNDPDDVVEFVGLHECGSR 100
101  VQVTDALVSTFLHSPPAGLSELRTRRREVPTECHVPRHSVNSDA 150
151  ILPFTVFPRTTALFEKLVFSLRREEDVGRKQSPFQLGDLALQREV 200
201  HIGSRIFLRITVDRCVATLTPDRIRLHKKVDFHGLVDGLYRSSHAK 250
251  APRPRPETLQTVVVEHFAKDSRITLYCTCHLKVTFADRVFDGLKACSE 300
301  IRSRKYVYVVEGSDICRCORRGSGLPGRSRRLSHLEGRVAKSVSHRM 350
351  RRIVTEAEITVGLLPLGRASDHGTEGSSPHTSVRLGLGLATVVSILTA 400
401  AITVAVLAKRIRHTEHPVLCFASVSD 427
    
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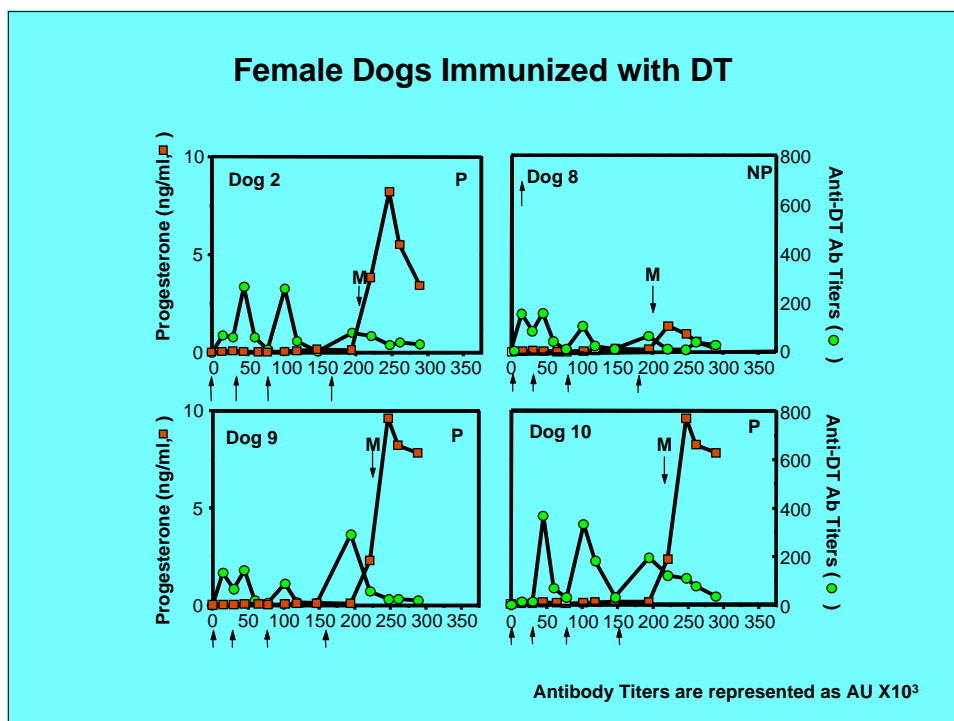
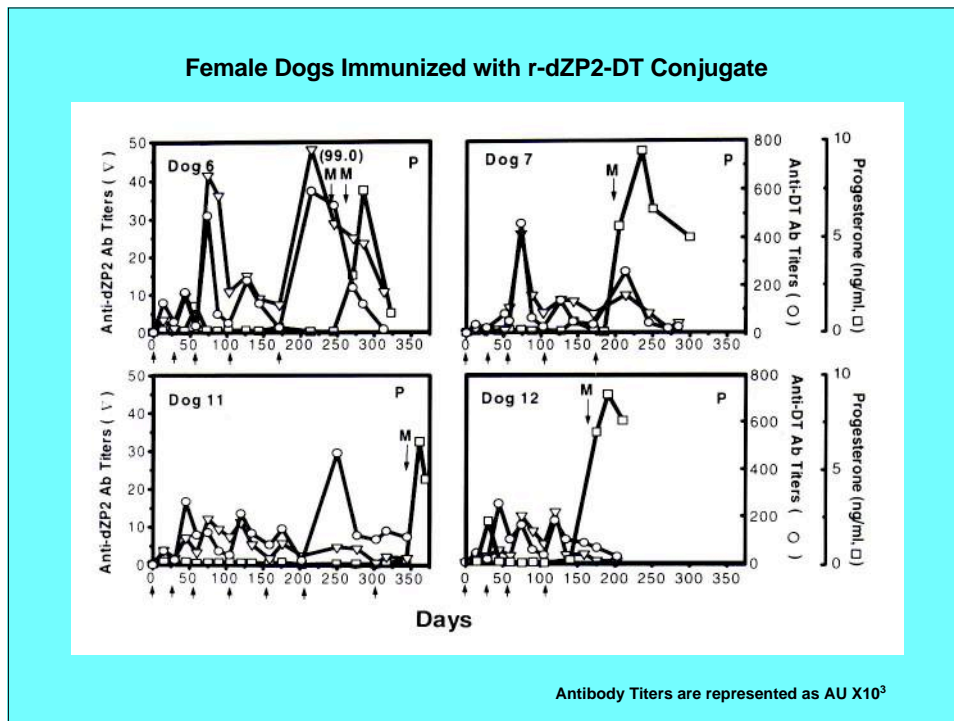
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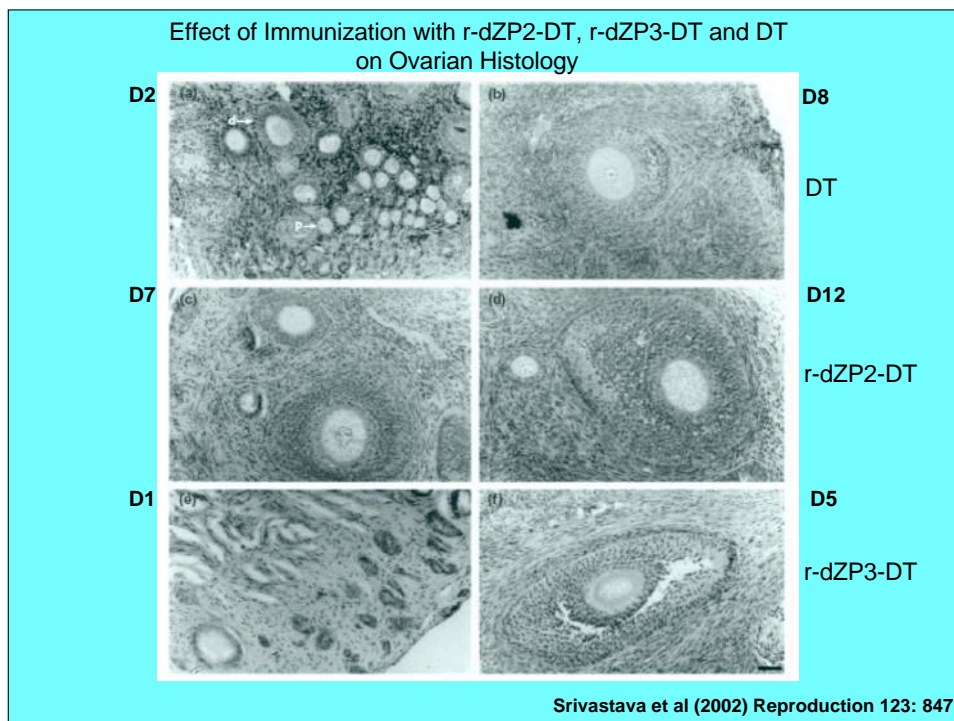
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Effect of Immunization with r-dZP3-DT, r-dZP2-DT and DT on Fertility in Female Dogs

Experimental group	Antibody titre range (AU X 10 ³)		Number of matings	Anti dZP3 titres at the time of mating	Pregnancy (Number of pups)
	dZP3	DT			
r-dZP3-DT immunized					
Dog 1	0.7 - 26.0	10.0 - 461.0	1	~ 6.0	NP
Dog 3	0.7 - 18.8	10.0 - 1760.0	1	~ 0.7	P (5)
Dog 4	0.8 - 12.8	20.4 - 608.0	3	~ 2.0	NP
Dog 5	0.7 - 93.1	11.4 - 1083.0	1	~ 8.8	NP
r-dZP2-DT immunized					
Dog 6	1.1 - 99.2	13.7 - 595.0	2	~ 99.0	P (4)
Dog 7	0.6 - 26.1	20.0 - 461.0	1	~ 5.0	P (6)
Dog 11	0.7 - 12.2	20.0 - 474.0	1	~ 2.0	P (4)
Dog 12	0.4 - 13.4	12.0 - 180.0	1	~ 2.1	P (3)
DT immunized					
Dog 2	nd	4.8 - 267.0	2	nd	P (4)
Dog 8	nd	4.6 - 153.0	1	nd	NP
Dog 9	nd	10.9 - 291.0	1	nd	P (5)
Dog 10	nd	12.8 - 365.0	1	nd	P (3)

nd: Not detected; P: Pregnant; NP: Non-pregnant

Srivastava et al (2002) Reproduction 123: 847

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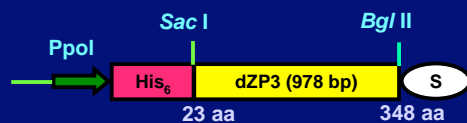
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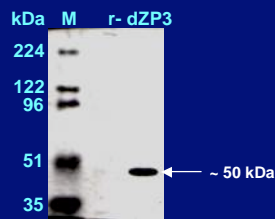
Female Dogs

Feasibility of Developing Immunocontraceptive Vaccine with Dual Efficacy to Inhibit Fertility and Provide Protection Against Rabies Infection

Cloning, Expression in Baculovirus and Purification of Recombinant Dog Zona Pellucida Glycoprotein-3 (r-dZP3)



pAcHLTA-dZP3

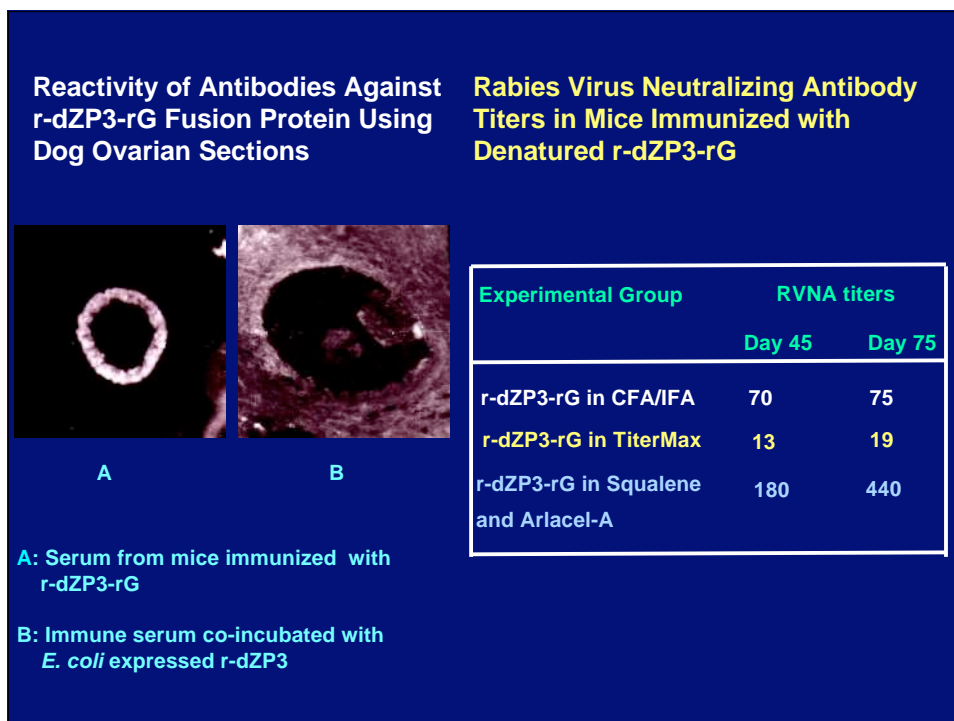
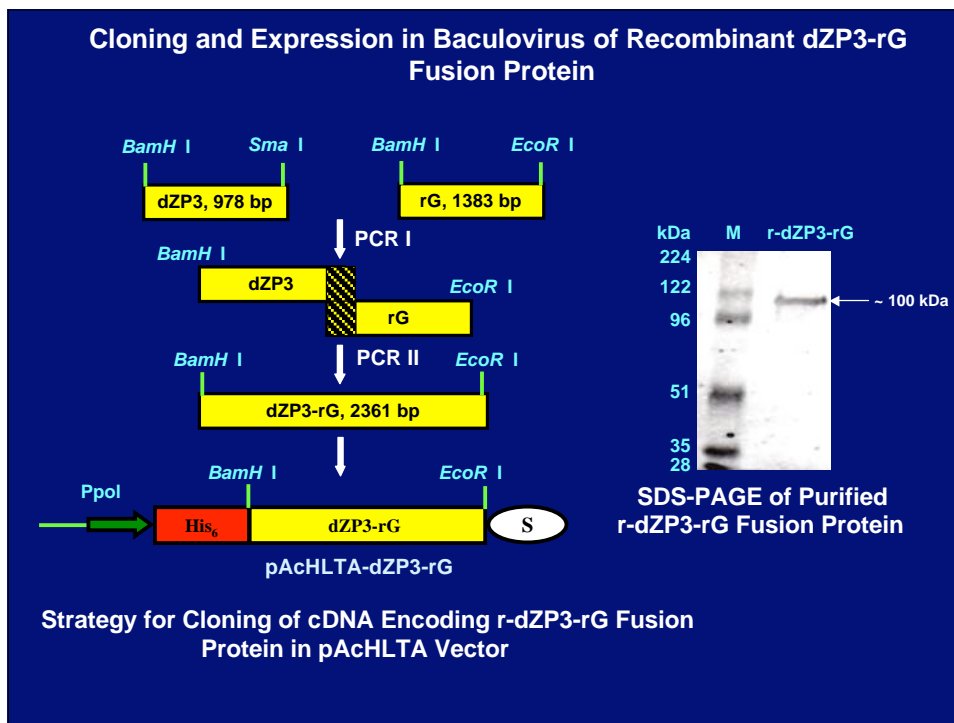


Western blot

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Comparison of antibody response in female mice immunized with denatured r-dZP3-rG using three different adjuvants in ELISA

Group	Experiment	Absorbance at 492 nm #	
		dZP3	rG
r-dZP3-rG in CFA/IFA	Preimmune (1 : 100) Day 0	0.59	0.35
	1st bleed (1 : 3200) Day 45	1.62	0.94
	2 nd bleed (1 : 3200) Day 75	2.04	0.95
r-dZP3-rG in TiterMax	Preimmune (1 : 100) Day 0	0.53	0.35
	1st bleed (1 : 3200) Day 45	0.54	0.13
	2 nd bleed (1 : 3200) Day 75	1.01	0.19
r-dZP3-rG in Squalene and Arlacel-A	Preimmune (1 : 100) Day 0	0.32	0.22
	1st bleed (1 : 3200) Day 45	1.71	1.23
	2 nd bleed (1 : 3200) Day 75	2.14	1.58

Figures in parentheses indicate the dilution of serum used in ELISA
Absorbance at 492 nm is the average value obtained from 5 mice/group

Summary of Active Immunization Studies in Nondescript Female Dogs

Experiment	Dogs came in heat	Number of matings	Antibody titers of 3 rd bleed by ELISA (10 ⁻³)			Progesterone value after mating (ng/ml)	Outcome of mating
			Anti-dZP3	Anti-rG	Anti-DT		
Immunized with r-dZP3-rG							
103	Yes	3	13.7	22.6	NA	2.00	NP
104	Yes	3	14.0	5.0	NA	5.60	P
105	-	-	4.0	4.0	NA	0.00	-
110	Yes	3	72.0	6.4	NA	2.50	NP
125	-	-	8.0	6.6	NA	0.04	-
122	Yes	3	11.5	20.0	NA	8.80	P
Immunized with r-dZP3-DT+rG							
101	Yes	3	10.9	7.9	169.5	1.50	NP
106	Yes	1	6.0	4.6	8.0	2.50	NP
124	-	-	16.7	11.7	8.2	1.09	-
126	-	-	4.0	4.0	10.5	1.48	-
Immunized with DT							
100	Yes	3	NA	NA	81.7	7.80	Aborted
115	Yes	3	NA	NA	67.7	6.50	Delivered (4)
116	Yes	1	NA	NA	92.2	6.00	P

The number of figures in parenthesis represents number of pups.
P, denotes for pregnancy and NP, denotes non-pregnant; NA-Not Applicable

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Salient Findings

Immunization with recombinant dog ZP3 demonstrated promise of curtailing conception in female dogs

Vaccine with dual efficacy meant for providing protection against rabies infection and curtailment of fertility needs further optimization with respect to the design of the immunogen and route of vaccine delivery

ALTERNATE APPROACHES

DNA vaccines

Direct gene transfer into mouse muscle *in vivo*
Wolff et al., Science 247, 1465-1468 (1990)

Genetic immunization is a simple method for eliciting an immune response
Tang et al., Nature 356, 152-154 (1992)

Advantages

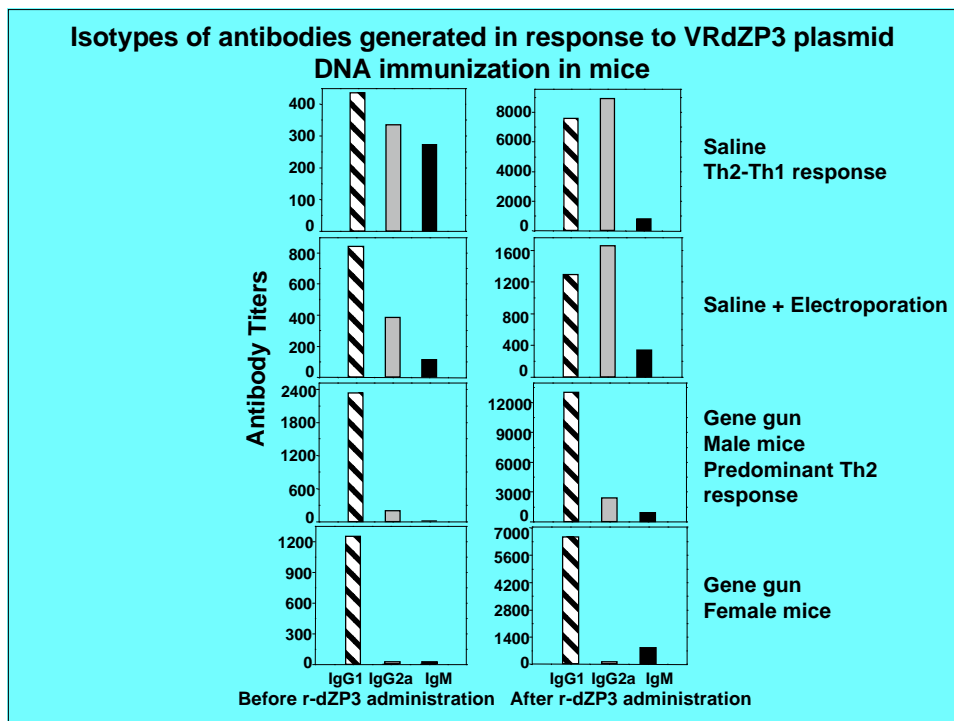
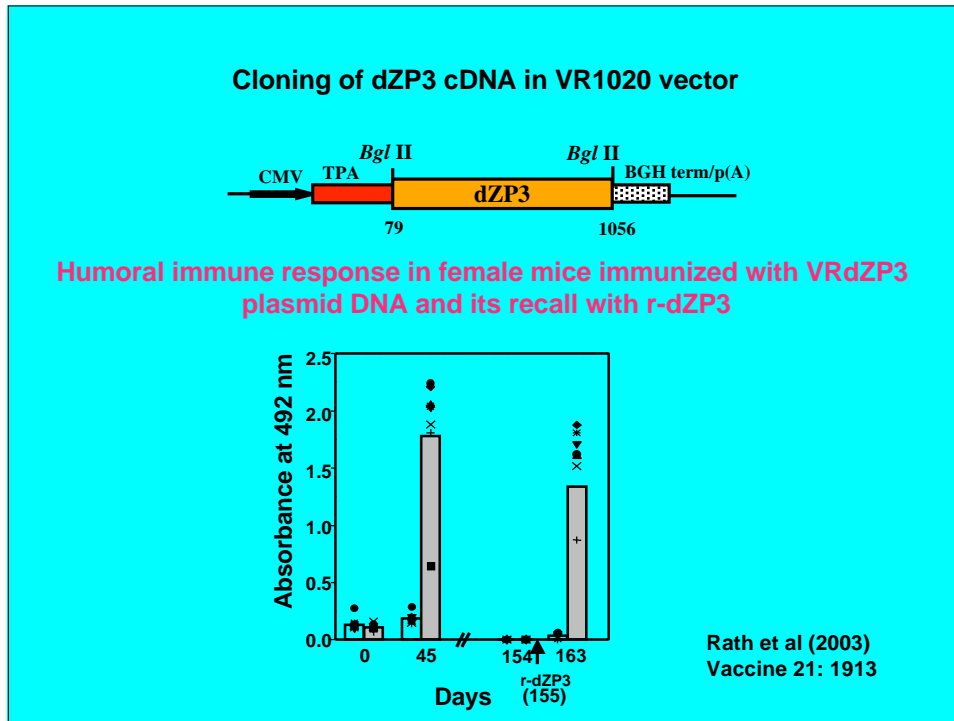
- Purity
- Amenable to genetic manipulation
- Stability
- Episomal nature
- Humoral and cell mediated immunity
- Long-lasting memory

Is it feasible to develop DNA vaccine
for immunocontraception?

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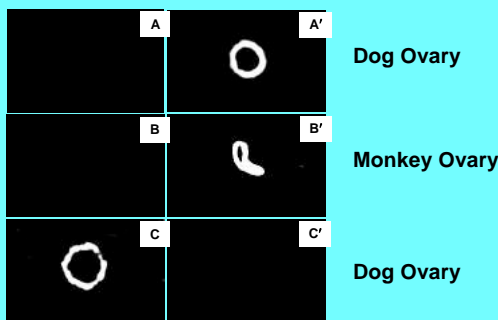


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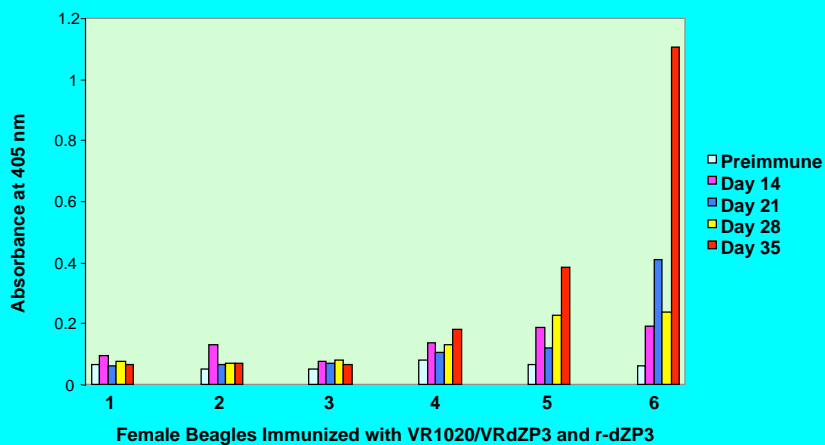
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Reactivity of the serum samples from female mice immunized with VRdZP3 plasmid DNA with dog and bonnet monkey ZP by indirect immunofluorescence



- A and B Immune sera from mice immunized with VR1020 vector
- A' and B' Immune sera from mice immunized with VRdZP3 plasmid DNA
- C Immunofluorescence with serum (1:2000 dilution) obtained from mouse immunized with VRdZP3 plasmid DNA
- C' Represents the same as in C but in the presence of r-dZP3 (1.0 µg/ml)

Humoral Immune Response in Female Beagle Dogs



One injection of VR1020/VRdZP3 plasmid DNA in saline (500 µg/animal)
 Boosted on day 21 with baculovirus expressed r-dZP3 (250 µg/animal) along with TiterMax.
 Beagles # 1-3 (control group)
 Beagles # 4-6 (experimental group)

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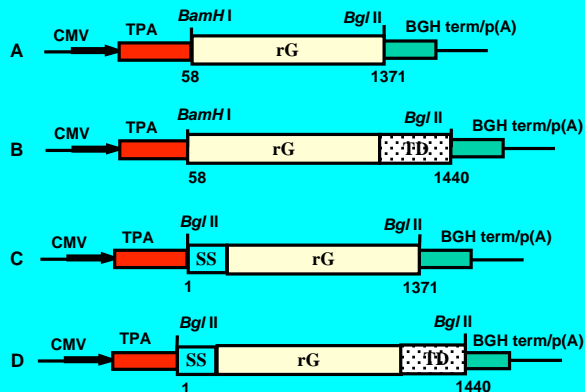
CONCLUSIONS

Plasmid DNA encoding dZP3 generates humoral immune response in both male and female mice. The antibodies thus generated recognize homologous and heterologous ZP.

Generation of an immune response in female mice and dogs provides further support to the fact that plasmid DNA immunization can break immunological tolerance, in this case, to a self protein.

A dominant anti-r-dZP3 IgG1 isotype response was generated by the gene gun delivery as compared to a mixed IgG1- IgG2a isotype response upon i.m. injection, showing the importance of the mode of delivery on the type of immune response elicited.

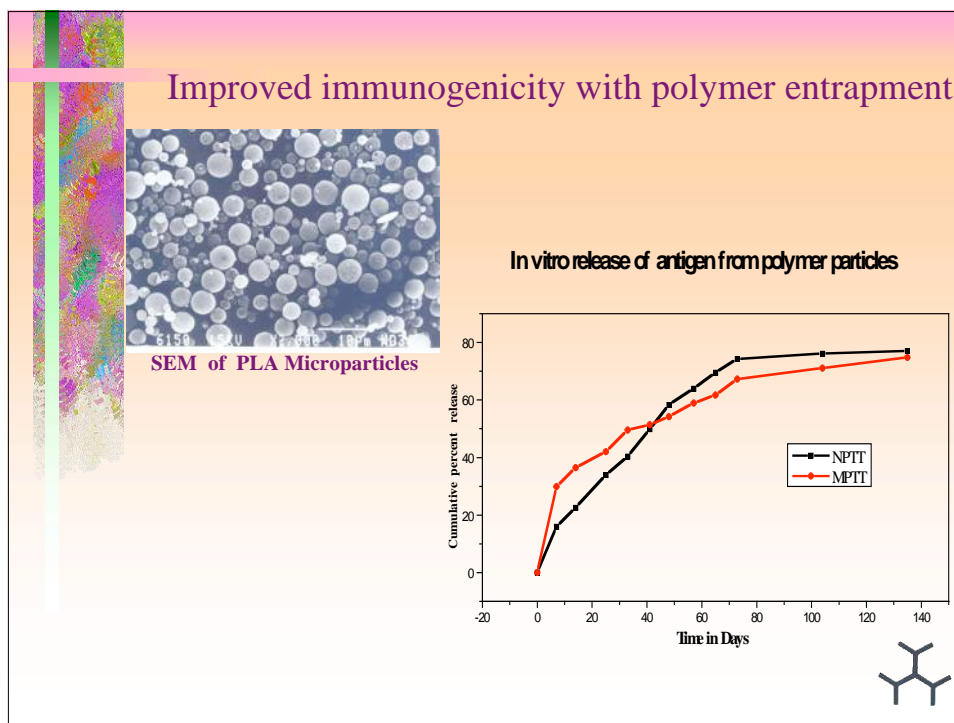
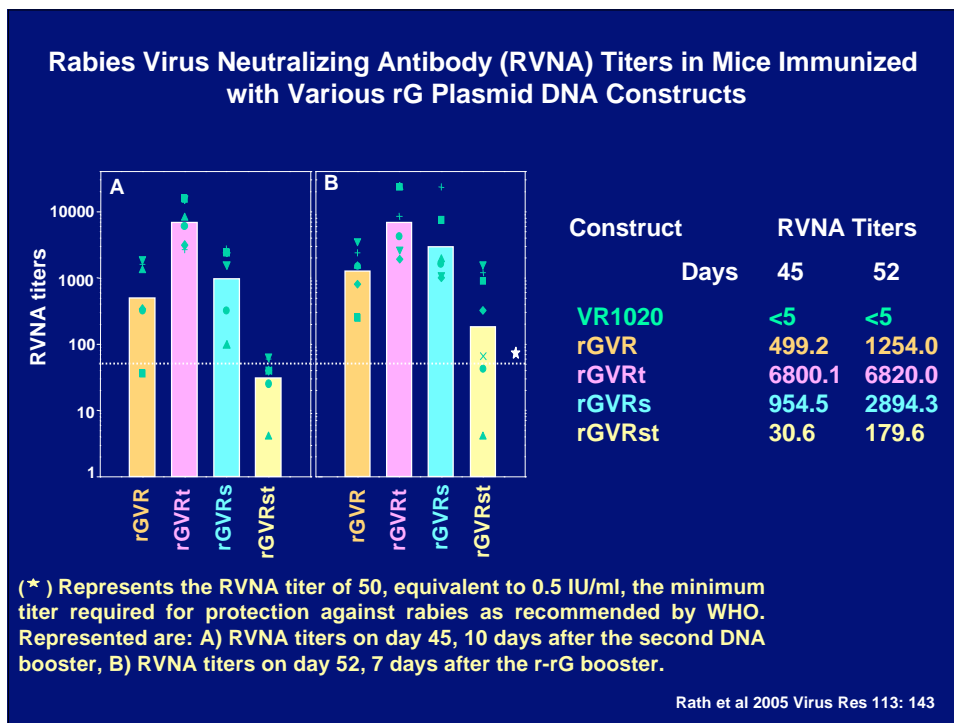
Schematic representation of four different plasmid DNA constructs encoding rabies virus glycoprotein (rG) cDNA



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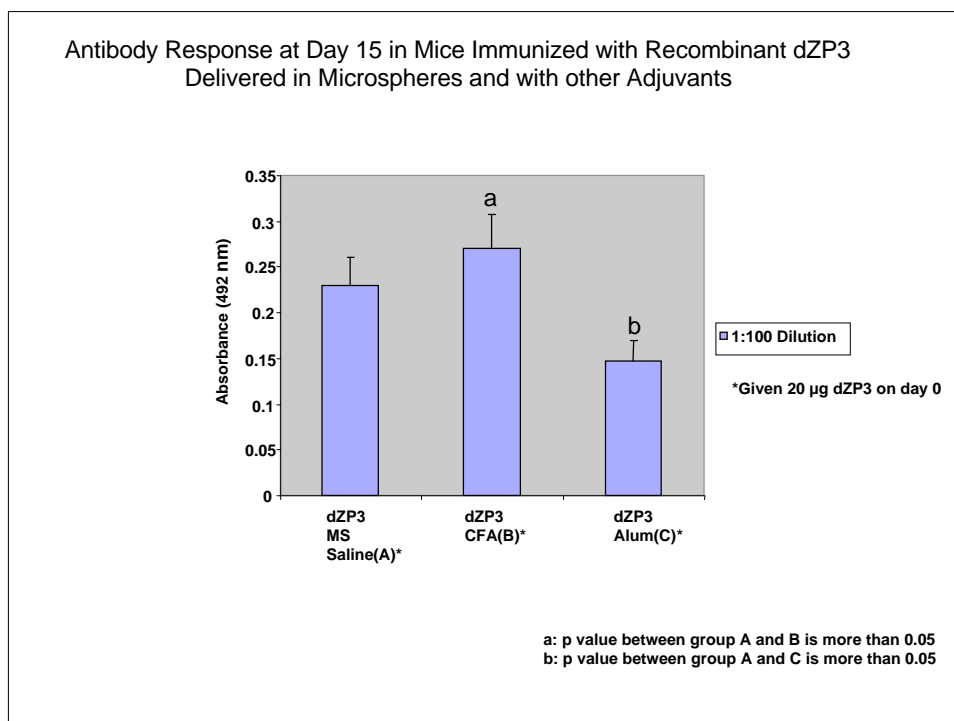
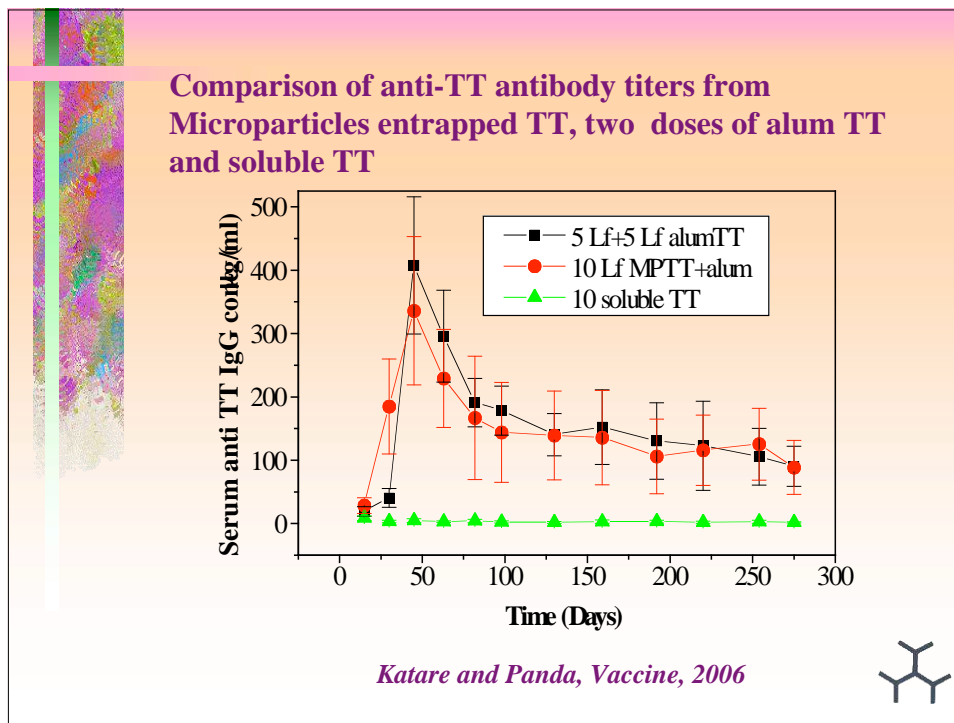
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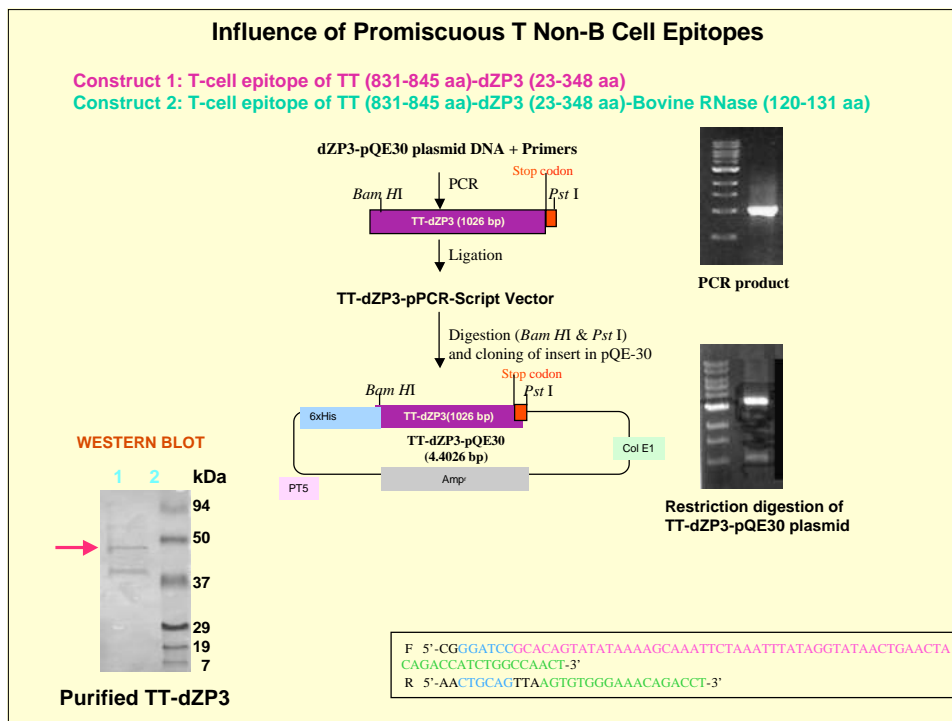
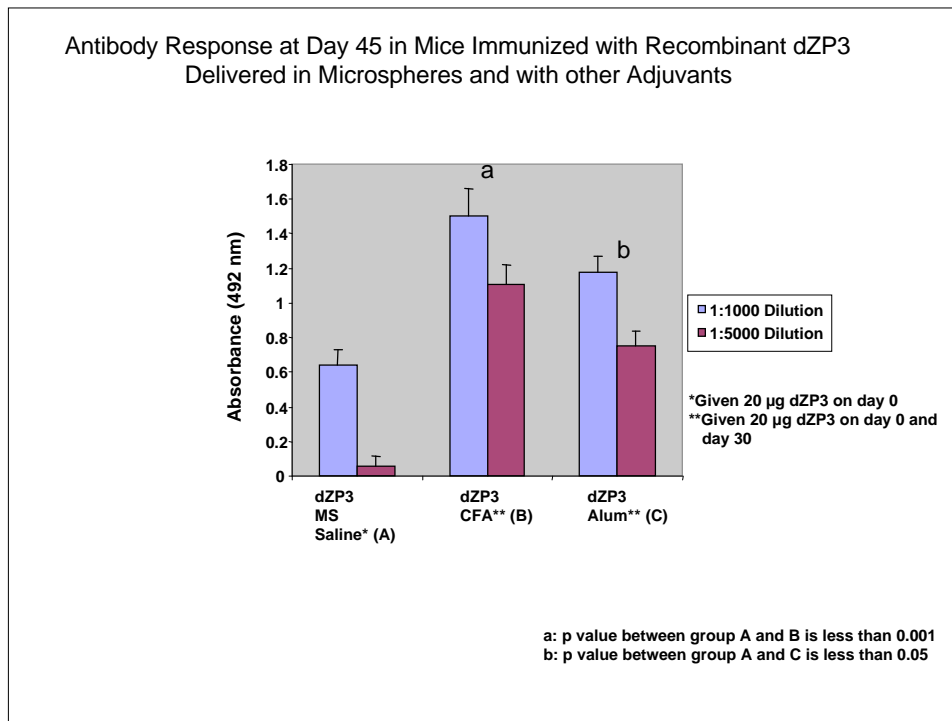
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FUTURE DIRECTIONS

WILDLIFE

- Increase the efficacy of immunocontraceptive vaccine by using multiple gamete antigens and/or hormones
- Delivery of vaccine (oral route)
- Safety protocols for vaccine delivery so as not to affect other species (humans in the context of Indian situation)
- Minimize the number of vaccine doses:
 - i) Different platforms for antigen presentation such as microspheres, Virus Like Particles (VLP), etc.
 - ii) Employment of potent adjuvants
 - iii) Improvement in the carrier
- Combination vaccine with other canine infectious diseases
- Combination vaccine with other diseases that are spread by canines to humans such as rabies (Presently working on the proposition of a combination of DNA Vaccine against rabies and recombinant dog ZP3)

Acknowledgements

Sangeeta Choudhury
Amulya K. Panda
Pankaj Suman
Neelu Srivastava
Archana Rath

International Collaborators

Charles E. Rupprecht
CDC, Atlanta, USA

Funding Agencies

Department of Biotechnology
Government of India