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Cloning, Expression, Purification and Contraceptive Efficacy Studies of a GnRH Receptor Based Recombinant Fusion Protein

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Abstract

The need for a contraceptive vaccine to control stray dog population is very essential in the current scenariowith increasing incidence of rabies and dog bites. It is seen as a viable non-surgical alternatemethod to spaying and castration.In the present study, a recombinant protein comprising of Gonadotropin Releasing hormone (GnRH) and Gonadotropin Releasing hormone receptor (GnRHR) were assembled along with the T cell epitopes of tetanus toxoid and canine distemper virus and was studied to explore its potential in contraception. The GnRH and GnRH receptor genes were successfully cloned into pET28a vector and expressed in BL21 DE3 bacterial expression system and further purified using Ni-NTA purification system.The resultant recombinant protein was expressed in *E. coli* to give rise to a 30kDa fusion protein. Further confirmation of the recombinant protein was done by western blot using Anti-His antibody. The mice were immunized with the GVAC09 fusion protein along with Freunds adjuvant. ELISA studies revealed the generation of a high antibody titre against the immunogen. *In vivo contraceptive studies* oftheGVAC09 immunized mice led to a decrease in litter size. From the current study with the proven immunocontraceptive potential of the recombinant protein, with further improvements GVAC09 recombinant protein has the potential to be a viable contraceptive vaccine to control stray dog population.

Keywords: Immunocontraception, vaccines, Gonadotropin Releasing hormone, Gonadotropin Releasing hormone receptor, stray dog population

Introduction

An over explosion of stray dog population in India and around the world is a cause for concern especially for developing and under developed countries because of the negative impact it has on public health and the economy of the country. Dogs are the main cause of rabies death with an estimated 59000 deaths per year worldwide (1,2), and in India an estimated 20000 people die due to rabies caused by dog bites which is 99% of the rabies cases (3,4) and hence controlling dog population can lead to reduction in rabies incidence (5). Stray dogs cause road accidents (6,7) and also kill small livestock which causes economic losses to the owners(8-10). Several private and government organizations have developed programs to control stray dog population. The primary methods employed in dog population control are culling and sterilization. Culling has been criticized as a temporary solution for a long-time problem (11) and is not as effective as sterilization (12) and the WHO has also passed guidelines to stop culling and to use alternative

methods. And hence sterilization is the only viable option to reduce stray dog population.

The current method employed in fertility control is surgical sterilization/ castration,which employs the catch-neuter- release principle(13). Surgical sterilization though highly effective and a permanent solution it cannot be carried out at thescale neededdue to problems faced by such sterilization programs such as the need for skilled veterinary surgeons, post-operative care expenses, hospital/ mobile van equipped with surgical instruments and medicines (10). To cope with these problems another method of sterilization called as immunocontraception has been developed and has proven to be effective in certain species such as boar, white-tailed deer and rocky mountain elk(14-16)].

GnRH is a decapeptide that is secreted by the hypothalamus and released into the bloodstream which then binds to the GnRH receptor. Nonetheless GnRH receptor (GnRHR) is a relatively new target that has not been explored much (17). GnRHR can be a very useful target for immunocontraception as it is an important link in the reproduction cascade. After GnRH is secreted, it is released into the blood stream, it then binds to the GnRHR that is present on the pituitary gland and results in the secretion of twovery important hormonesFollicle stimulating hormone (FSH) and Luteinizing hormone (LH) which are essential for reproduction. Since it is present in both sexesit is a natural target for immunocontraception(18).

GnRH based contraceptive vaccines work by inducing the production of GnRH neutralizing antibodies also called anti-GnRH antibodies(19). These anti-GnRH antibodies bind to the receptor and inactivate endogenous GnRH and also prevent them from binding to the receptor (20). This results in inadequate levels of GnRH which further suppress gonadal function and therefore can lead to population control (21,15). However, to develop an effective GnRH based vaccine is challenging because GnRH owing to its small size is a weak immunogen (20). In order to overcome this weak immunogenicity several strategies have been developed such as, the use of T cell epitopes (22,23), conjugating carrier proteins such as chitosan(24), KLH(15), and the use of different adjuvants like Adjuvac a *Mycobacterium* based adjuvant (19,25). GnRH based contraceptive vaccines have been developed for various species (25,26). Gonacon is one such vaccine which has been effective in many species such as cats, black tailed prairie dogs, white tailed deer and wild horses (27,28, 29).

In the present work, GnRH and GnRH receptor (GnRHR) were cloned into pET 28a expression system to express a recombinant protein which has not been reported thus far and hence was attempted. The study was aimed to enhance the contraceptive potential of the GnRHR based vaccines. The purified GVAC09 recombinant fusion protein and its effectiveness in contraception was studied in mice. The results of the present study throw some light towards thedifficulties in the development of GnRH based contraceptive vaccines.

Materials and Methods

Reagents:

The enzymes NcoI and XhoI, T4DNA Ligase, PCR reagentsand Unstained protein marker were procured from Thermo Fisher Scientific. Plasmid extraction and Gel purification kit were purchased from G Biosciences. IPTG, prestained marker, Nuvia IMAC resin were obtained from Bio-Rad.

Cloning of synthetic gene

GnRH and GnRHR coding sequence were retrieved from NCBI. The sequences were assembled along with the T cell epitopes of tetanus toxoid and canine distemper virus. Codon optimization and de novo synthesis were carried out by GenScript Biotech and cloned into pUC57 cloning vector.The gene GVAC09 was sub cloned into pET28a expression vector using forward primer V04FP- 5'- CCA TGG GGA

TGG CGA GCG CGA G -3' and reverse primer V09RP- 5'- CTC GAG ATT CAG GTT GCT CTG GTG CAG C -3'. The reaction mixture composed of3µL of 10X PCR buffer, 3µL $MgCl_2$ (20 mM), 1.0µL for each dNTP (2.5mM), 2.0 µLGVAC09 plasmid DNA, 1.0µL of each primer and 1 µL of Pfu DNA polymerase (5 units/ µL) in a total volume of 30µL. The program for PCR was one cycle at 95°C for 5 minutes, 30 cycles with denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, followed byextension for 1 minute at 72°C and final extension at 72°C for 5 minutes. The NcoI- GVAC09- XhoI amplified fragment was purified,digestedwithNcoI and XhoI restriction enzymes and then ligated to pET28a vector. The GVAC09-pET28a plasmid was transformed into Top 10 competent cells for plasmid amplification by heat shock method. The selection of the transformed GVAC09-pET28a plasmid was carried out on Luria-Bertani (LB) agar medium with 100 µg /ml kanamycin.

Successful transformation was confirmed by colony PCR. A single positive clone was selected and incubated in 20ml LB broth with 100µg /ml kanamycin and incubated overnight. The GVAC09 plasmid was then isolated using G Biosciences plasmid isolation kit and was successfully transformed into BL21 DE3 cells. The selection of the transformantswas done on LB agar medium containing 100µg /ml kanamycin. Colony PCR was carried out for the confirmation of clones consisting of the GVAC09 gene. The bacterial stocks were stored at -80ºC in 20% (v/v) glycerol for long time storage.

Table 1 Primers used for cloning and PCR

Gene Con-struct	Primer Name	Primer Sequence 5'-3'
GVAC09	V04FP	CCATGGGGATGGCGAG-CGCGAG
	V09RP	CTCGAGATTCAG-GTTGCTCTGGTGCAG
	T7 Forward	TAATACGACTCAC-TATAGGG
	T7 Reverse	GCTAGTTATTGCTCAG-CGG

Expression of GVAC09 recombinant protein

A single colony containing GVAC09 gene was inoculated in 10ml LB broth containing 100µg /ml kanamycin and incubated overnight at 37ºC in a shaker incubator (180rpm). 1ml of overnight culture was transferred into a 1000ml Erlenmeyer flask with 200ml LB broth containing 100µg /ml kanamycin and was incubated at 37ºC with 180rpm shaking. The growth rate of the cells was monitored every hour spectrophotometrically at 600nm till the OD values reached 0.6. Further, the cells were induced with IPTG to a final concentration of 1mM. 2 ml of the same culture was collected as un-induced sample or negative control before adding IPTG. The incubation of the culture was carried out in a shaker incubator (180rpm)for 6 hours at 37ºC. After incubation, harvesting of the cells was done by centrifugation at 7500rpm for 15 mins at 4ºC.

The re-suspension of the cells wasdone in 20ml of lysis buffer that contained 8M urea, 1mM PMSF, 300mM NaCl, 50mM Na_2HPO_4at pH 8.0. The cells were then placed in an ice jacket and sonicated for 15 mins with 10 seconds burst cycle. After sonication the contents were centrifuged at 10000rpm for 15mins. The supernatant was collected and added to Ni-NTA IMAC resin column and incubated with shaking for one hour at 4ºC. Further, washing of the column was carried out to remove non-specific proteins that may be loosely bound to the resin. Elution of the GVAC09 protein was carried out by adding elution buffer containing 300mM Imidazole. The elution fraction was collected and desalted by dialysis. The concentration of urea in the dialysis buffer was gradually reduced from 7M to 0M urea and finally dialyzed against PBS.

The purified protein was characterized by SDS PAGE. Confirmation of the recombinant protein was done by western blotting with the use of anti-His monoclonal antibody.

Mice

Six- to eight-week-old pathogen free Balb/c mice having a body weight of 25-30g were purchased from Geniron Biolabs, Bangalore, India and divided into three groups at random and named Group A,B and C. Each group had 6 male and 6 female mice. Two groups A and B served as treatment groups and Group C was the control group. Male and female mice of each group were housed separately in polycarbonate cages and maintained at appropriate temperature and humidity conditions.The animal studies were carried out in accordance with the *Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines (CPCSEA No. 493/GO/ReBiBt-S/Re-L/01)*after necessary approvals.

Immunization of mice

After one week of acclimatization, both male and female mice were subcutaneously administered with purified GVAC09 recombinant protein. Briefly, Group A mice were immunized with 100µg and Group B mice were immunized with 200µg of purified GVAC09 recombinant protein mixed with equal volumes of Freund's complete adjuvant (Sigma). The mice in control group were immunized with normal saline. The Group A and group B mice were boosted with 100 and 200µg of purified GVAC09 recombinant protein and Freund's incomplete adjuvantrespectively on day 21.

Table 2 Study design

Group	Immunogen/Saline	No. of mice	Dose	Route of administration	Adjuvant
Group A	GVAC09 recombinant protein	6 male+ 6 female	100µg	Sub cutaneous	FCA- Primary FIA- Booster
Group B	GVAC09 recombinant protein	6 male+ 6 female	200µg	Sub cutaneous	FCA- Primary FIA- Booster
Group C Control	Saline	6 male+ 6 female	100µl	Sub cutaneous	

Humoral immune response to GVAC09 recombinant protein

The mice were anesthetized prior to blood collection and blood was collected by retro-orbital bleeding before primary immunizationand on day 14, 28and 45 respectively post immunization to determine the antibody titres. The serum reaction against GVAC09 recombinant protein was determined by indirect ELISA. Briefly, 200ng/well of purified GVAC09 recombinant protein in carbonate buffer was coated on microtitre ELISA plates. Phosphate buffered saline with 0.05% Tween 20 (PBST) was used for washing the plates and the plates were subsequently blocked with PBS containing 3% Bovine Serum Albumin (BSA). The washing of the plates with PBST was followed by the addition of serum samples diluted in 0.1% BSA to the wells and were subsequently incubated at 37ºC for about 1.5 hours. Further washing of the plates was carried out using PBST followed by the addition of Goat- anti-mouse IgG conjugated with horseradish peroxidase (Jackson immunoresearch) and the plates incubated at 37ºC for 1 hour. The plates were once again washed with PBST and the plateswere developed with tetramethylbenzidine (TMB), the reaction stopped with $H_2SO_4$and the absorbance measured at 450 nm. The data is represented as antibody titre, the endpoint titer was defined as the reciprocal of the highest dilution that gives a reading above the cutoff which was set as an absorbance of 1.0. Theimmune response producedby the mice in

the groups are represented as arithmetic mean + standard error (S.E.) of the antibody titers of the individual animals as previously described by Gupta N, 2016.

Mating studies

On the day after the collection of the last bleed i.e 46th day, immunized male and female mice were mated with unimmunized female and male mice whose fertility was proven. Each immunized male mouse was housed with two unimmunized female mice in one cage and two immunized female mice was housed with one male mice of proven fertility in one cage to test for *in vivo* contraceptive efficacy. The mice were monitored daily to check for the formation of vaginal plugs in female mice which indicate mating. Once mating is confirmed the male mice were removed and each female mice with confirmed vaginal plug were housed separately.

Statistical analysis

The results obtained in the experimental groups of immunized animals treated with purified GVAC09 recombinant protein to inhibit fertilization was statistically analyzed and the results obtained are expressed as mean+ standard deviation.

Results and Discussion

Cloning of synthetic gene

The genes encoding GnRH and GnRHR along with the antigenic epitopes were synthesized and cloned into pUC57 cloning vector to form pUC57-GVAC09 plasmid. Specific primers were designed to amplify the GVAC09 gene with NcoI and XhoI restriction sites and the amplified gene was sub-cloned into pET28a expression system and labeled as pET28a-GVAC09 plasmid. Double digestion method was used for the confirmation of the integrity of the pET28a-GVAC09 recombinant vector using NcoI and XhoI restriction enzymes (Fig. 1). Thetransformation of pET28a-GVAC09 plasmid into competent BL21DE3 *E. coli* cellswas successful with an efficiency of over 70%. Agarose gel electrophoresis of the colony PCR products revealed an800bp bandcorresponding to the size of GVAC09 gene.

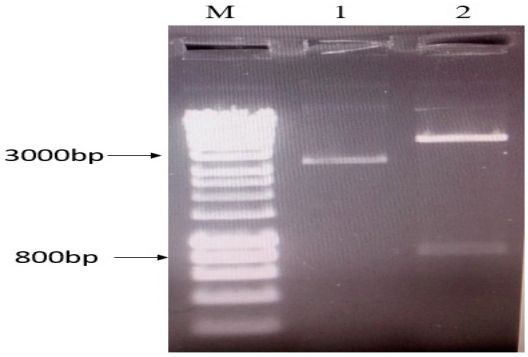

Fig.1 Agarose gel electrophoresis showing restriction digestion of pET28a-GVAC09 plasmid

Expression of GVAC09 recombinant protein

After verification of the cloning of GVAC09 gene into pET28a vector, the resultant recombinant plasmid was transformed into BL21DE3 *E. coli* cells successfully. Upon induction with 1mM IPTG and subsequent incubation for 6 hours, the cells were centrifuged and sonicated. Purification of the crude GVAC09 recombinant protein was achieved by Ni^{2+}-NTA affinity purification. The characterization of the GVAC09 recombinant protein by SDS PAGE analysis showed a band at 30kDa which is consistent with the theoretical mass of the protein (Fig 2). Western blotting using Anti-His antibody (Bio-Rad) as the primary antibody confirmed the expression of GVAC09 recombinant protein (Fig 3). The purification yield was 60mg protein per 1L of bacterial culture.

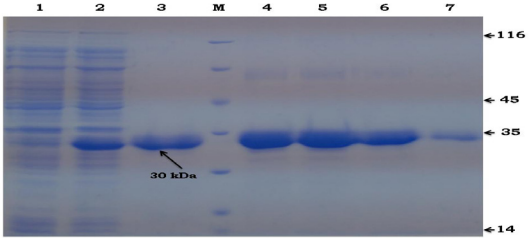

Fig.2 SDS PAGE analysis of GVAC09 (purified recombinant protein)

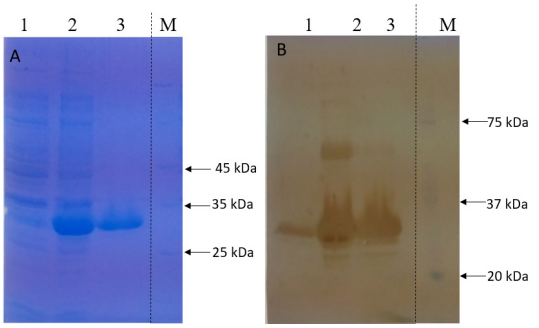

Fig.3A.SDS PAGE. **B**. Western blot analysis of GVAC09 recombinant protein

Humoral immune response

The Group A and B mice were immunized with GVAC09 recombinant protein and Group C mice were administered normal saline according to the study design (Table 2).

Immunization of mice with GVAC09 recombinant protein led to the generation of anti-GVAC09 antibodies which was determined by indirect ELISA. The mean antibody titre of Group A mice immunized with 100µg GVAC09 recombinant protein was found to be 25.33±2.37 x 10^3AU, 40.0±5.3 x 10^3 AU and 99.25± 4.8 x 10^3 AU on day 14, 28 and 45 respectively. The antibody titres of Group B mice immunized with 200µg GVAC09 recombinant protein were 29.33±1.79 x 10^3AU, 56.4±4.4 x 10^3 AU and 102.05± 9.7 x 10^3 AU on day 14, 28 and 45 respectively. Immunization of Group B mice with 200µg of GVAC09 recombinant protein led to a higher antibody titre when compared to mice immunized with 100µg GVAC09 recombinant protein on day 14, 28 and 45.

Mating studies

Mating studies in mice were conducted to check for the contraceptive efficacy of GVAC09. 46 days post immunization ofGVAC09 recombinant protein the immunized male and female mice were paired with unimmunized female and male mice respectively. The mean litter size of Group Amice administered with 100µgGVAC09 and the Group B mice administered with 200µg GVAC09 were 9.83+ 0.79 and 9.58+ 1.03 respectively, whereas for Group C, the control group it was observed as 12+ 1.29. The number of pups delivered by immunized female mice and the unimmunized female mice mated with immunized male mice were significantly lower as compared to untreated control group

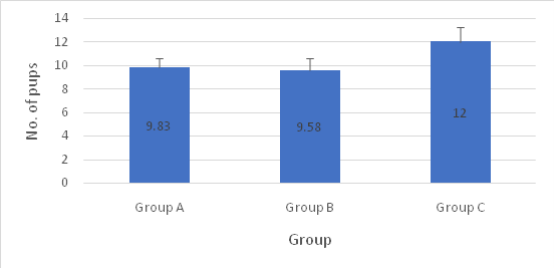

Fig.4 Mean litter size

The need for a contraceptive vaccine which is safe and effective has been there for a very long time. Several approaches have been made to develop a contraceptive vaccine; one such approach is called as immunocontraception [30]. An immunocontraceptive vaccine works by eliciting an immune response either humoral or cell mediated against targets which are

Table 3 Antibody titre of mice immunized with GVAC09 recombinant protein

Group	Immunogen	Concentration	Antigen used for ELISA	Antibody titre (x10^3)		
				Day 14	Day 28	Day 45
Group A	GVAC09	100µg	GVAC09	25.33±2.37	40.0±5.3	99.25±4.8
Group B	GVAC09	200µg	GVAC09	29.33±1.79	56±4.4.0	102.05±9.7

essential for reproduction (31). Itobstructs the biological roles of the reproductive hormones responsible for reproduction (32).The two major targets for a contraceptive vaccine are GnRH and Zona pellucida, GnRH is responsible for the biosynthesis and release of FSH and LH which in turn facilitate oogenesis and spermatogenesis leading to the formation of the oocytes and sperm respectively. Therefore, an immune response against the sperm or the egg will interfere with the process of fertilization. One such GnRH based vaccine called GonaCon™ has been successfully used to control the population of White- tailed deer and wild horses (25). Alternative vaccines for immunocontraception are being developed to increase the efficiency and duration of the antifertility effect (33). Alternatively, GnRH receptor is a relatively new and vital target for contraception due to its location outside of the blood brain barrier which makes it easily accessible. GnRH receptor is also crucial in the hypothalamus-pituitary-gonadal cascade of reproductive events that can lead to infertility.Further any hinderance in the binding of GnRH to the GnRH receptor inhibits reproduction (17).

The aim of this study was to check for the contraceptive efficacy of GVAC09, a recombinant protein containing GnRH, GnRH receptor genes along with helper epitopes from Tetanus toxin and canine distemper virus. Similarly, tetanus epitopes have been used in order to induce CD4+ helper T lymphocytes (HTLs) responses in a vaccine for brucellosis(34). A major advantage of using GnRH as a target for contraception is GnRH is present in both males and females and hence a single vaccine is sufficient to administer both male and femaledogs. The benefits of using T helper epitopes have been well documented when vaccines against FMD and CSF were developed (35). The GnRH and GnRH receptor genes were successfully cloned into pET28a vector and expressed in BL21 DE3 bacterial expression system and further purified using Ni-NTA purification system. The resultant recombinant protein GVAC09along with Freunds adjuvantwas then immunized in male and female mice, after which specific immune response against GVAC09 recombinant protein was elicited which was determined by ELISA. Adjuvants other than Freunds adjuvant has shown notable decrease in ovarian pathology(36).

The antibody titre against GVAC09 recombinant protein was found to have reached its highest peak after the booster dose was administered. It should be observed that the interval between the First dose and the booster dose is very crucial as a long or short interval between the two doses may lead to a weak immune response (37).

In our previous studies we have studied the immunocontraceptive potential of a GnRH and GnRHR based vaccine fused along with zona pellucida coding sequence (38), the results of which are very similar to the results obtained in the current study. This shows that GnRH and GnRHR alone can give a similar contraceptive effect as compared to the two being fused with zona pellucida. The average antibody titres of Group A and Group B did not vary significantly, similar to the results obtained in our previous studies (38). GVAC09 recombinant protein induced a robust immune response which resulted in the generation of antibodies against GnRH and GnRH receptor which in turn resulted in a decline in litter size of mice administered with GVAC09 recombinant protein in comparison to the litter size of the control group mice. Although there was areduction in litter size, complete contraception was not achieved,which could be due to the fact that the antibody titre might have reduced dramatically between the time the mice were paired for mating and mating actually taken place (39). The decrease in litter size observed when a GVAC09 recombinant protein immunized mice was mated with an unimmunized partner is very crucial in a dog population control program as it might not be possible to immunize every individual dog. Therefore, GVAC09 recombinant fusion protein with further improvement, that may result to

have a longer lasting antibody titre, could lead to complete reduction in litter size and can eventually be used clinically as a contraceptive vaccine for dog population control.

Conclusion

To conclude, we have developed an immunocontraceptive vaccine candidate by cloning GnRH and GnRH receptor genes in pET28a vector and expressed the recombinant protein GVAC09 in BL21DE3 *E. coli* expression system. The GVAC09 recombinant protein was administered in mice to assess its efficacy in contraception. A reduction in litter size was observed in mice immunized with GVAC09 recombinant protein in comparison with the control group. Hence with slight improvements GVAC09 can be used as a viable contraceptive vaccine candidate. Studies with respect to the effect of GVAC09 recombinant protein on the reproductive organs will shed some light on further improving the efficacy of the vaccine candidate.

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