Vaccine 37 (2019) 910–914

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Short communication

Development of an auxotrophic, live-attenuated *Brucella suis* vaccine strain capable of expressing multimeric GnRH

G.P. Smith [a], N. Jain-Gupta [a], H. Alqublan [a], E.M.S. Dorneles [b], S.M. Boyle [a], N. Sriranganathan [a,*]

[a] Center for One Health Research, Virginia Maryland College of Veterinary Medicine, Virginia Tech, Blacksburg, VA 24061, USA
[b] Departamento de Medicina Veterinária, Universidade Federal de Lavras, Lavras, MG, Brazil

ARTICLE INFO

Article history:
Received 2 August 2018
Received in revised form 12 December 2018
Accepted 30 December 2018
Available online 14 January 2019

Keywords:
Brucella suis
Feral swine
Live-attenuated vaccine
Immuno-contraception

ABSTRACT

Feral swine cost around $1.5 billion each year in agricultural, environmental, and personal property damages. They are also the most widespread carriers of the zoonotic disease brucellosis, which threatens both livestock bio-security and public health. Currently, there is no approved vaccine against brucellosis in pigs. This is a preliminary report on the development of a live-attenuated *B. suis* vaccine that could be employed to deliver heterologous antigens to control swine populations. An attenuated vaccine strain provided significant protection against *B. suis* challenge in mice. Leucine auxotrophy in the vaccine strain allowed the over-expression of heterologous antigens without the use of antibiotic resistant markers. Vaccinated mice showed the development of antibodies against expressed antigen. Further evaluation is required to assess its ability to cause infertility using the mouse model prior to further testing for use as a tool for feral swine population and disease control.

1. Introduction

Brucellosis, caused by members of the Gram negative bacterial genus *Brucella*, is among the most prevalent zoonotic diseases worldwide [1]. In the United States, the disease has been essentially eradicated from domestic swine and cattle herds; however, the disease still exists in wildlife reservoirs including elk (*Cervus elaphus*), bison (*Bison bison*), and feral swine (*Sus scrofa*). Among these, feral swine are the most widespread and they have been attributed to recent outbreaks of the disease in domestic herds as well as zoonotic infections in humans [2,3]. The population of feral swine, which are an invasive species in the U.S., has roughly quadrupled in the last decade and they account for roughly $1.5 billion in damages annually nationwide [4,5]. They have been shown to carry *B. suis*, which has a host preference for swine but can also infect cattle, as well as *B. abortus* that has a host preference for cattle. All *Brucella* spp. carried by feral swine are also capable of causing infection in humans [2]. Thus, feral swine not only cause widespread economic loss, but their increased contact with humans and domestic livestock raises their potential to spread brucellosis, among other zoonotic diseases such as leptospirosis and influenza [6].

Currently, there is no approved vaccine against swine brucellosis. This work describes development of a rough strain of *B. suis*, VTRS2, which has defined deletion mutations in genes *wbo*A (encoding glycosyl transferase) and *leu*B (encoding isopropyl malate dehydrogenase). Deletion of *wbo*A confers the rough phenotype, while deletion of *leu*B renders the strain unable to synthesize leucine to grow in a leucine-deficient environment. This auxotrophy allows the strain to maintain the antibiotic markerless family of plasmids, pNS4, which complement the *leu*B gene [7] and can be used to overexpress heterologous and homologous antigens in VTRS2. One such antigen is the candidate immunocontraceptive; multimeric GnRH (mGnRH).

Immunocontraceptive vaccines have been proposed as an additional tool for wildlife management agencies to control nuisance species [8,9]. Current vaccines stimulate an immune response against endogenous reproductive hormones such as gonadotropin releasing hormone (GnRH) conjugated to an antigenic carrier [8]; however, most of these vaccines are delivered as subunit preparations that are not cost effective to produce and would be highly expensive to use in wild pigs [10]. Herein, an attenuated *B. suis* vaccine was engineered to express mGnRH, a small recombinant antigenic form of GnRH originally developed by the Talwar Research Institute (New Delhi, India), for expression in *Escherichia coli* and purification for subunit delivery [10–12]. Strain VTRS2 was then characterized in the mouse model to establish whether the strain is sufficiently attenuated *in vivo* for use as a candidate vaccine

* Corresponding author.
E-mail address: nathans@vt.edu (N. Sriranganathan).

https://doi.org/10.1016/j.vaccine.2018.12.070
0264-410X/

and to demonstrate if the vaccine can protect mice against virulent *B. suis* challenge. In addition to strain VTRS2, strain VTRS2 expressing the immunocontraceptive peptide mGnRH was also tested for the ability to elicit a significant anti-mGnRH IgG response in mice.

2. Materials and methods

2.1. Ethics statement

All mouse experiments were done in our AAALAC approved facility and the experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) (protocol # CVM-12-144) at Virginia Tech. For *retro*-orbital bleeding, mice were anaesthetized under isofluorane using Vet Equip Mobile Laboratory Animal Anesthesia System. Mice were euthanized using an overdose of carbon dioxide followed by cervical dislocation.

2.2. Creation and characterization of the candidate vaccine strain VTRS2

Strain VTRS2 was created via sequential deletion of 416 bp of *wbo*A (encoding a glycosyl transferase, LPS O-side chain polymerization gene) and 574 bp of *leu*B (encoding isopropyl malate dehydrogenase) from the virulent reference strain *B. suis* 1330 using cre-lox recombination methodology as previously described by Rajasekaran et al. [7]. The gentamicin resistance marker was replaced with the chloramphenicol resistant marker found in plasmid pGEM3Z

Clones were screened for roughness by crystal violet staining and clumping in the presence of acriflavine and the serum agglutination test [13,14]. The deletion in *leuB* was confirmed by the lack of growth in leucine-deficient *Brucella* minimal medium (BMML). Complementation by pNS4 plasmids was phenotypically confirmed by the restoration of growth in BMML medium [7,15].

2.3. Over-expression of multimeric GnRH (mGnRH) in strain VTRS2

To create the immunocontraceptive vaccine strain, codon optimized mGnRH sequences was cloned into plasmid pNS4 and transformed into strain VTRS2 to generate VTRS2-pNS4-mGnRH (Fig. 1a). Expression of mGnRH in strain VTRS2 was determined using standard SDS-PAGE and Western blot techniques [16]. Briefly, strains VTRS2-pNS4 and VTRS2-pNS4-mGnRH were grown in BMML for 48 hrs and spun down to obtain a pellet. Pellets were resuspended in 40 μL of 10 mM tris-HCl and lysed in a boiling water bath for 15 min and then mixed 1:1 with Laemmli SDS-PAGE sample buffer for SDS-PAGE electrophoresis. After electrophoresis, the separated proteins were transferred onto a nitrocellulose membrane (0.45 μM). Anti-His-HRP antibody serum (Abcam) was used to detect his tagged mGnRH protein via hydrogen peroxide-based development and exposure to radiographic film.

2.4. Clearance and challenge study

To evaluate the attenuation of the VTRS2 candidate vaccine strains, four to six week old female BALB/c mice (Harlan Laboratories) were inoculated intraperitoneally (IP) with $5x10^5$ CFUs (colony-forming units) of *B. suis* 1330, VTRS2-pNS4 or VTRS2-pNS4-mGnRH. Each VTRS2 group of mice was euthanized and processed at weeks 4 and 6 (n = 5). Additional 1330 groups were similarly processed at 8 and 14 weeks. At euthanasia, total splenic CFUs were determined by plating serially diluted spleen homogenates on tryptic soy agar (TSA). Lowest limit of detection of CFU is Log_{10} 1.3 and in cases in which no bacterial colonies were seen in the lowest dilution Log_{10} 1.3 was recorded as the CFU count.

For the challenge study, 4 to 6 week old female BALB/c mice were administered $\sim 5 \times 10^5$ CFUs IP of either strain VTRS2-pNS4 or VTRS2-pNS4-mGnRH (n = 10), an additional group was administered sterile saline. At six weeks post-vaccination, all mice were either boosted with 5×10^4 CFUs or challenged with 5×10^4 *B. suis* 1330 (n = 5). Boosted mice were challenged two weeks after the booster vaccination. All mice were euthanized two weeks post-challenge and total splenic CFUs determined by serial dilution. Vaccine strain and challenge strain were differentiated to have a rough and smooth phenotype respectively using neutral acriflavin and crystal violet staining. Blood was collected *retro*-orbitally at weeks 0, 4, and 6 post-vaccination and at the time of euthanasia.

2.5. Anti-mGnRH ELISA

mGnRH antibody response using purified mGnRH was assessed via enzyme-linked immunosorbent assay (ELISA). Purified mGnRH was obtained from pRSETb-mGnRH grown in *E. coli* DH10b and purified using Ni-His affinity chromatography (Qiagen). Elated mGnRH was adsorbed to wells of polystyrene plates (Nunc Maxisorp) at the protein concentration of 1.0 μg/well in 50 μL of bicarbonate buffer (pH 9.6). After blocking the wells with 2% bovine

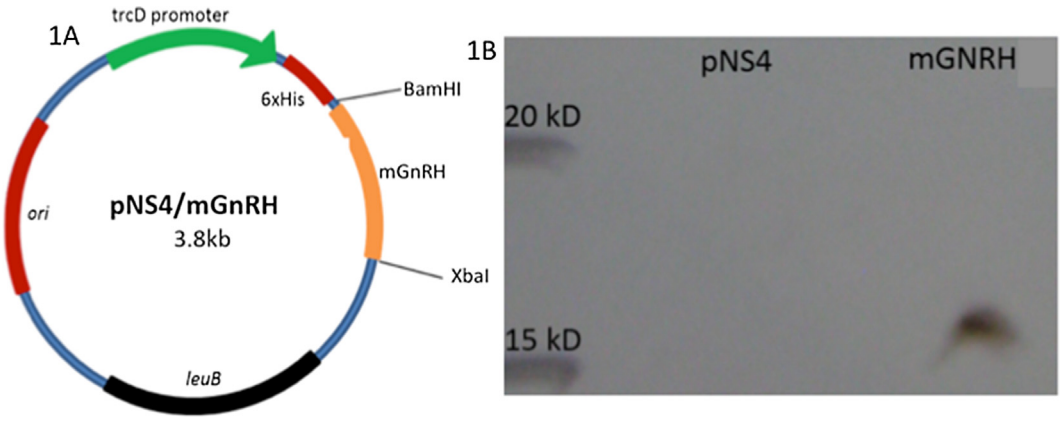

Fig. 1. a. **Schematic showing the plasmid vector pNS4-mGnRH:** A 3.8kB plasmid vector with *trcD* promoter was used to over-express the mGnRH cloned between *BamHI* and *XbaI* restriction sites. The vector provides a his-tag at the N terminal of the cloned gene and complements the l*euB* deficiency in the auxotrophic vaccine strain VTRS2. **1b. Heterologous protein expression in strain VTRS2 using plasmid vector pNS4-mGnRH:** Western blot of extracted protein lysates from strain VTRS2 with empty expression vector pNS4 (pNS4) and strain VTRS2-pNS4-mGnRH shows the presence of the 16–17 kDa mGnRH antigen (mGNRH) using anti-His-tag-HRP antibody (Invitrogen). The blot was developed using light emission on a radiographic film.

serum albumin in phosphate buffered saline 0.01 M, pH 7.2, 0.05% Tween 20, plates were washed and incubated for 3–4 h with plasma (1:100 dilution). All the samples were tested in triplicates. After incubation, plates were washed and incubated with 1:2000 horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Invitrogen). Plates were developed using TMB and stopping the reactions using 0.18 M sulfuric acid. Absorbance was measured at 450 nm using a microtiter plate reader.

2.6. Statistical analysis

All splenic titers were compared using Student's *t*-test to compare means between individual groups. All statistical analysis was performed using the JMP Pro 11 software (SAS). ELISA results were analyzed using 2-way ANOVA.

3. Results

3.1. Strain VTRS2 is rough and unable to synthesize leucine and grow without complementation

Strain VTRS2 was characterized phenotypically to confirm its rough morphology and the inability to grow without leucine supplementation in BMML medium. Strain VTRS2 was confirmed to have rough colony morphology via crystal violet staining, acriflavine staining, and serum agglutination. The colonies behaved similarly to strain RB51 for all 3 tests; strain VTRS2 takes up crystal violet, clumps in the presence of neutral acriflavine, and fails to agglutinate when incubated with hyper-immune serum against the smooth *Brucella* LPS. Furthermore, the strain was shown to be unable to grow on leucine deficient minimal media without complementation of *leu*B on a pNS4-based plasmid. In tryptic soy broth, no differences in growth kinetics were observed between the reference strain *B. suis* 1330 and VTRS2 (data not shown).

3.2. Over-expression of heterologous antigens

Western blot (Fig. 1b) performed using anti-His antibodies shows the presence of a protein band corresponding to mGnRH (16.4 kD) in the strain VTRS2-pNS4-mGnRH compared to the control strain.

3.3. Clearance and protection in mice

At four weeks post-inoculation, all strain VTRS2 inoculated mice had between 2.3 and 2.6 Log_{10} reduction (>97%) in total splenic CFUs versus control mice challenged with virulent *B. suis* 1330 ($P < 0.001$, Fig. 2). At six weeks, all strain VTRS2 inoculated mice had >3.8 Log_{10} reduction (>99.98%) in total splenic CFU versus controls and had reached the minimum limit of detection (1.3 Log_{10}) and were considered cleared. There was no difference in titer between samples plated on enriched agar versus leucine-deficient agar suggesting the pNS4 plasmid was maintained *in vivo*. Mice vaccinated with strain VTRS2-pNS4-mGnRH or VTRS2-pNS4 showed a reduction of 0.47 and 0.64 Log_{10} in total splenic CFUs respectively compared to unvaccinated controls (Fig. 3a) when challenged with virulent *B. suis* 1330. Compared to the controls, mice that received primary and booster dose of VTRS2-pNS4-mGnRH or VTRS2-pNS4 vaccine strains showed a reduction of Log_{10} 0.6 ($P < 0.05$) and Log_{10} 1.25 ($P < 0.001$) respectively. Presence of vaccine strain (rough phoentype) was ruled out using acriflavin agglutination test and crystal violet staining that showed only smooth strain of *Brucella* was obtained from the mice after euthanasia. All groups were significantly different from unvaccinated controls and strain VTRS2 with the control plasmid pNS4 was significantly more protective than strain VTRS2-pNS4-mGnRH ($P < 0.01$) (Fig. 3b).

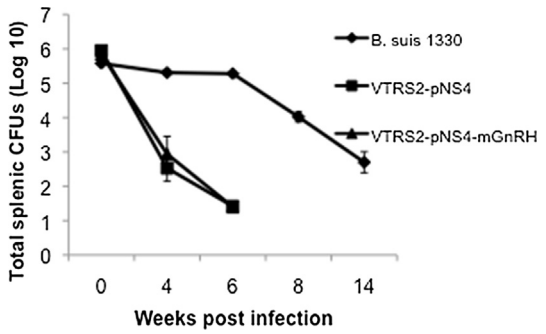

Fig. 2. Clearance of strain VTRS2 Vaccine Strains from BALB/c Mice: All mice received an initial dose of ~5×10^5 CFUs. Splenic titers in all vaccinated mice were significantly lower than the virulent strain control mice at all time-points measured and all vaccinated mice reached the lower limit of detection (20 CFUs/ml of splenic homogenate, 1.30Log_{10}) by 6 weeks post-inoculation.

3.4. Strain VTRS2-pNS4-mGnRH elicits a significant anti-mGnRH immune response

Peak mGnRH-specific antibody titers occurred at four weeks post-vaccination and titers were significantly ($P \leq 0.05$) higher in strain VTRS2-pNS4-mGnRHb vaccinated versus strain VTRS2-

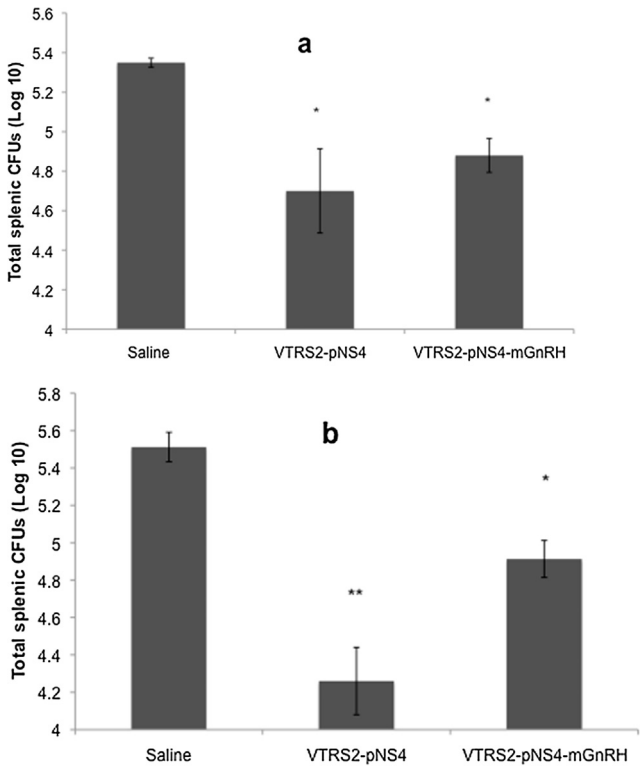

Fig. 3. Protection in mice against *B. suis* challenge: (a) Splenic titer of *B. suis* 1330 challenge strain two weeks post-challenge in mice vaccinated with strains VTRS2-pNS4, VTRS2-pNS4-mGnRH, or sterile saline. Asterisk * means statistically significant difference ($P < 0.05$) compared to the non vaccinated mice. (b) Splenic titer of *B. suis* 1330 challenge strain two weeks post-challenge in mice vaccinated with strains VTRS2-pNS4, VTRS2-pNS4-mGnRH, or sterile saline that received a booster vaccination six weeks after initial inoculation. Asterisk * means statistically significant difference ($P < 0.05$) compared to the non vaccinated mice and double asterisk ** means statistically significant difference ($P < 0.05$) from both unvaccinated as well as strain VTRS2-pNS4-mGnRH vaccinated mice.

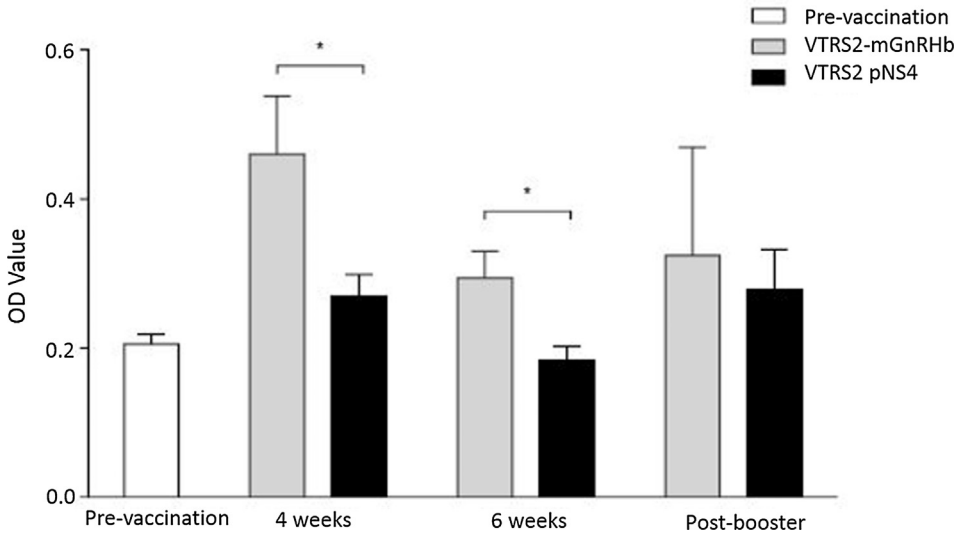

Fig. 4. IgG titers in mice post vaccination: Mouse anti-mGnRH IgG Response at serum dilution 1:100 at 0, 4, and 6 weeks post-vaccination and 2 weeks following booster vaccination. The coating-antigen used was purified mGnRH peptide and antibody binding was detected using peroxidase-conjugated anti-mouse IgG. Strain VTRS2-pNS4-mGnRH showed significantly higher antibody titers (shown by asterisk *, $P < 0.05$) compared to VTRS2-pNS4 vaccinated mice at week 4 and 6 post vaccination.

pNS4 vaccinated mice at four and six weeks post-vaccination (Fig. 4).

4. Discussion

The candidate vaccine strain VTRS2 was successfully created and shown to have several characteristics desirable of a brucellosis vaccine intended for use in feral swine. Rough colony morphology due to a *wbo*A deletion mutation is a known attenuating feature of *Brucella* and is considered the major mutation in the most widely used vaccine strain in the United States, strain RB51 [17]. However, RB51 is a strain of *B. abortus*, and has not been shown to be effective against swine brucellosis in controlled studies [18]. Despite this, a rough vaccine is essential in countries with brucellosis surveillance programs, as smooth strains interfere with such programs by causing sero-conversion in vaccinated animals. Thus, a rough *B. suis* strain makes a logical candidate for an effective vaccine in swine. Deletion mutation of *wbo*A has previously performed [19]; however, the mutagenesis procedure used involved permanent insertion of kanamycin resistance, which is undesirable, especially in a vaccine with potential use in wildlife *Brucella* reservoirs such as feral swine. Moreover, clearance between four and eight weeks is desirable for a live *Brucella* vaccine to ensure adequate cell-mediated immunity is generated without allowing an unacceptable infection to become established from the vaccine strain. Our data shows that strain VTRS2 is attenuated *in vivo* and evaluation of the vaccine strains for protection against challenge was pursued accordingly. There was no difference in titer between splenic samples plated on TSA versus leucine-deficient minimal medium. This demonstrates the stability of the pNS4 family of plasmids *in vivo*. Furthermore, presence of the antigen-delivery plasmid pNS4-mGnRH did not affect clearance kinetics of strain VTRS2 in mice but did influence the immunity against experimental infection.

Recently, a rough *B. suis* strain was isolated by Stoffregen et al. (2013) from a feral pig that has shown promise as a candidate vaccine [20]. While promising, the strain is early in its testing and characterization and the exact mutations are unknown. Furthermore, it lacks the *leu*B deletion mutation present in strain VTRS2, which allows for antigen delivery without the use of antibiotic resistance markers using the pNS4 family of plasmids. Our results suggest that plasmids containing recombinant heterologous antigen can be maintained and their antigens expressed in VTRS2. Strain VTRS2 expressing mGnRH was able to elicit a significant IgG immune response against the mGnRH antigen at four and six weeks post-inoculation.

From these studies, it can be concluded *B. suis* strain VTRS2 is attenuated *in vivo*, provides protection against *B. suis* challenge and heterologous antigens could be maintained and expressed. Strain VTRS2-mGnRH could be a useful tool to add to the available resources of wildlife management agencies to combat the growing problem of feral swine over- population. Further testing to characterize the strain for its efficacy in protecting against virulent *B. suis* challenge in pigs and its ability to cause fertility defects are warranted.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgement

This research project was funded by Virginia APHIS-USDA (422319).

References

[1] Seleem MN, Boyle SM, Sriranganathan N. Brucellosis: A re-emerging zoonosis. Vet Microbiol 2010;140:392–8.
[2] Stoffregen WC, Olsen SC, Jack Wheeler C, Bricker BJ, Palmer MV, Jensen AE, et al. Diagnostic characterization of a feral swine herd enzootically infected with *Brucella*. J Vet Diagn Invest 2007;19:227–37.
[3] Gelfand MS, Cleveland KO, Buechner D. Neurobrucellosis in a Hunter of Feral Swine. Infectious Diseases in Clinical Practice. 2014;Publish Ahead of Print:10.1097/IPC.0000000000000160.
[4] Pimental D. Environmental and economic costs of vertebrate species invasions into the United States; 2007.
[5] Sandfoss MR, DePerno CS, Betsill CW, Palamar MB, Erickson G, Kennedy-Stoskopf S. A serosurvey for *Brucella suis*, classical swine fever virus, porcine circovirus type 2, and pseudorabies virus in feral swine (Sus scrofa) of Eastern North Carolina. J Wildl Dis 2012;48:462–6.
[6] Meng XJ, Lindsay DS, Sriranganathan N. Wild boars as sources for infectious diseases in livestock and humans. Philos Trans Royal Soc B: Biol Sci 2009;364:2697–707.
[7] Rajasekaran P, Seleem MN, Contreras A, Purwantini E, Schurig GG, Sriranganathan N, et al. *Brucella abortus* strain RB51 leucine auxotroph as an

environmentally safe vaccine for plasmid maintenance and antigen overexpression. Appl Environ Microbiol 2008;74:7051–5.
[8] Killian G, Miller L, Rhyan J, Doten H. Immunocontraception of florida feral swine with a single-dose GnRH vaccine. Am J Reprod Immunol 2006;55:378–84.
[9] Miller LA, Gionfriddo JP, Fagerstone KA, Rhyan JC, Killian GJ. The single-shot GnRH immunocontraceptive vaccine (GonaCon™) in white-tailed deer: comparison of several GnRH preparations. Am J Reprod Immunol 2008;60:214–23.
[10] Miller LA, Talwar GP, Killian GJ. Contraceptive effect of a recombinant GnRH vaccine in adult female pigs. In: Proceedings-Vertebrate Pest Conference, 2006. p. 106–9.
[11] Talwar GP, Raina K, Gupta JC, Ray R, Wadhwa S, Ali MM. A recombinant luteinising-hormone-releasing-hormone immunogen bioeffective in causing prostatic atrophy. Vaccine 2004;22:3713–21.
[12] Gupta JC, Raina K, Talwar G, Verma R, Khanna N. Engineering, cloning, and expression of genes encoding the multimeric luteinizing-hormone-releasing hormone linked to T cell determinants in *Escherichia coli*. Protein Expr Purif 2004;37:1–7.
[13] Marx CJ, Lidstrom ME. Broad-host-range cre-lox system for antibiotic marker recycling in gram-negative bacteria. Biotechniques 2002;33:1062–7.
[14] Schurig GG, Roop Ii RM, Bagchi T, Boyle S, Buhrman D, Sriranganathan N. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol 1991;28:171–88.
[15] Rajasekaran P, Surendran N, Seleem MN, Sriranganathan N. Schurig GG, Boyle SM. Over-expression of homologous antigens in a leucine auxotroph of *Brucella abortus* strain RB51 protects mice against a virulent *B. suis* challenge. Vaccine 2011;29:3106–10.
[16] Vemulapalli R, He Y, Buccolo LS, Boyle SM, Sriranganathan N, Schurig GG. Complementation of *Brucella abortus* RB51 with a functional *wboA* gene results in O-antigen synthesis and enhanced vaccine efficacy but no change in rough phenotype and attenuation. Infect Immun 2000;68:3927–32.
[17] Vemulapalli R, McQuiston JR, Schurig GG, Sriranganathan N, Halling SM, Boyle SM. Identification of an IS711 element interrupting the wboA gene of *Brucella abortus* vaccine strain RB51 and a PCR assay to distinguish strain RB51 from other *Brucella* species and strains. Clin Diagn Lab Immunol 1999;6:760–4.
[18] Stoffregen WC, Olsen SC, Bricker BJ. Parenteral vaccination of domestic pigs with *Brucella abortus* strain RB51. Am J Vet Res 2006;67:1802–8.
[19] Winter AJ, Schurig GG, Boyle SM, Sriranganathan N, Bevins JS, Enright FM, et al. Protection of BALB/c mice against homologous and heterologous species of *Brucella* by rough strain vaccines derived from *Brucella melitensis* and *Brucella suis* biovar 4. Am J Vet Res 1996;57:677–83.
[20] Stoffregen WC, Johnson CS, Olsen SC. Immunogenicity and safety of a natural rough mutant of *Brucella suis* as a vaccine for swine. Res Veterinary Sci 2013;95:451–8.