Heliyon 7 (2021) e06149

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Brucella abortus RB51 Δ*leuB* expressing *Salmonella* FliC conjugated gonadotropins reduces mouse fetal numbers: A possible feral swine brucellosis immunocontraceptive vaccine

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ARTICLE INFO

Keywords:
Fertility control
Feral swine
FSHβ/GnRH
Brucella
RB51
Salmonella
Feral swine population control

ABSTRACT

Population and health management of wildlife is a key to environmental health, domestic herd health, and ultimately public health. Many different methods including: surgical sterilization, poison baits, and sponsored hunting programs have been used in the attempt to control populations of various nuisance animal species. Particular interest has been given to immunocontraception through wildlife vaccination protocols. This study specifically looked at the potential immunocontraceptive and protective properties of a *Brucella abortus* RB51 Δ*leuB* vaccine expressing *Salmonella typhimurium* FliC conjugated to porcine follicle stimulating hormone beta subunit (FSHβ) or gonadotropin releasing hormone (GnRH) DNA sequences. *B. abortus* RB51 Δ*leuB* pNS4-*TrcD-FliC- FSH*$_\beta$ (RB51LFSHβ) and *B. abortus* RB51 Δ*leuB* pNS4-*TrcD-FliC-GnRH* (RB51LGnRH) were tested in a pilot breeding study with BALB/c mice, and a significant reduction in fertility characteristics was observed in both male and female mice. Ultimately, this study provides support to test these vaccine candidates in feral swine, a destructive invasive species in the United States of America.

1. Introduction

Of the invasive species affecting the United States of America (USA), *Sus scrofa,* the feral pig is of particular interest to the United States Department of Agriculture (USDA) for population control. In fact, the US government has allocated millions of dollars to the cause [1]. The current measures of trapping, hunting, and poison baiting have not been effective enough to control their spread. This species causes about $1.5–2.5 billion in agricultural damage a year in the USA, and feral swine continue to thrive in almost every state [2, 3, 4]. Not only do these prolific breeders pose a threat to the agricultural economy, but they also pose a threat to domestic herds and public health. Feral swine are known carriers of over 67 different zoonotic disease causing agents including *Brucella* spp., African Swine Fever, pseudorabies, and hemorrhagic *E. coli* [5, 6, 7, 8, 9]. In fact, there have been multiple human illnesses confirmed to be from feral swine interactions [9]. Of particular importance is the causative agent of brucellosis, *Brucella* spp. *Brucella* spp. seroprevalence in feral swine populations has been reported as high as 50% in parts of the USA, and they could easily transmit the disease to unvaccinated domestic animals or hunters [9]. Thus, there is a need for a vaccine that can both reduce fertility and prevent the spread of swine brucellosis.

The USDA Animal and Plant Health Inspection Services (APHIS) has set specific criteria for the creation of an ideal immunocontraceptive wildlife vaccine. The vaccine must: be safe for the target species, be free of undesirable side effects, not spill over and affect non-target species, not prevent the target species from being safe to consume, cause little social effect on the target species, and produce a long-term but reversible infertility [10]. Many components of the reproductive system have been targeted to try and achieve these specific criteria, but the search for strong vaccine candidates continues. The current front-runners are the porcine zona pellucida (PZP) and gonadotropin-releasing hormone (GnRH) targeting vaccines [10]. In particular, the USDA's multimeric-GnRH vaccine, GonaConTM, has been approved and used in multiple wildlife species to control their populations. Improvac/Improvest®, another GnRH based vaccine, has been successfully approved for use in the swine industry to prevent boar taint [11]. GnRH, when conjugated with highly immunogenic antigens, has proven to be successful in the interruption of fertility allowing these two subunit vaccines to become an industry standard [11, 12, 13]. The vaccine candidates evaluated in this study provide advantages for use in feral swine including: culturability, forgoing the expensive purification process of

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https://doi.org/10.1016/j.heliyon.2021.e06149
Received 3 June 2020; Received in revised form 15 September 2020; Accepted 27 January 2021

subunit vaccines, and the possibility for administration of the vaccine using oral bait.

GnRH is a small peptide hormone that is produced by the hypothalamus and regulates the production of pituitary gonadotropins; luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In simple terms, these hormones have receptors throughout the body and are vital in the production of sperm and testosterone in the male, along with follicular development, ovulation, and pregnancy in the female. Ultimately, GnRH is at the top of this hypothalamic-pituitary-gonadal axis and stimulates the production of LH and FSH, both leading to many sex-specific fertility characteristics. FSH in the male stimulates the Sertoli cells in the testis and the maturation of spermatids. In the female, FSH nurtures the developing follicle to become an ovulatory follicle. FSH is a vital hormone in successful reproduction and is composed of two subunits (α and β) [14, 15]. The β subunit has variable homology between species and is more important in the receptor binding process [16]. This fact could provide a potentially safer immunocontraceptive target that could be designed to more specifically target a species in field applications.

To evaluate if the hypothalamic-pituitary-gonadal axis has been negatively affected there are various fertility characteristics that can be measured. In veterinary medicine, specific methods of quantifying these characteristics are part of a breeding soundness exam attempting to determine the ability of an animal to reproduce. Some of these parameters include sperm motility, sperm morphology, sperm concentration, testicular size, hormone levels, birth/pregnancy rates, fetal viability, and anatomical conformation [14]. Before analysis of abnormal, 'normal' fertility characteristics need to be defined. BALB/c inbred mice have been used in research since 1923 and substantial data on their reproductive characteristics exists. In addition, these characteristics are closely monitored and reported by research strain suppliers. Their average litter size is between five and seven pups, and they are uniformly considered good breeders with a long reproductive life stage [17, 18]. One report in the literature opposes this view showing a 32% infertility rate, but the circumstances of the matings are not well described. The large majority of the literature reports between 0-10% infertility rates [17, 19]. Primiparous monogamous pairs in particular have been reported to have 100% fertility rates [19]. Non-viable fetuses per litter are reported on average between 1 and 2 fetuses [19]. The epididymal sperm concentrations in male BALB/c mice have been reported to be between $15.5 \times 10^3 \pm 4.4$ and $7.67 \times 10^6 \pm 1.41$ sperm/mL at around 80 days of age [20, 21, 22, 23]. BALB/c males have been reported to have testes weighing between 90mg and 212mg [18, 26].

Ultimately, there is a need for an immunocontraceptive vaccine that can be tailored to specific wildlife species for population control. With the threat to domestic herd and public health, along with the agricultural economy, by feral swine a dual-purpose vaccine preventing the spread of the population and zoonotic diseases is ideal. This study reports a leucine auxotroph of *B. abortus* RB51 expressing gonadotropins conjugated to *Salmonella typhimurium* FliC reduces fertility in a murine model. This data, plus previously published data from our lab, justifies further testing of these vaccine candidates in feral swine [27, 28].

2. Material and methods

2.1. Bacterial strains

Brucella abortus RB51 Δ*leuB* (RB51L), *B. abortus* RB51 Δ*leuB* pNS4-*TrcD-FliC-porcineFSHbeta* (RB51LFSHβ), and *B. abortus* RB51 Δ*leuB* pNS4-*TrcD-FliC-GnRH* (RB51LGnRH) from the culture collection at Virginia-Maryland College of Veterinary Medicine (VMCVM) (Blacksburg, VA). An *Escherichia coli* strain HB101 (Sigma Aldrich) containing the pNS4 plasmid constructs from the bacterial culture collection at VMCVM was used for cloning purposes [25]. Bacterial strains were grown at 37 °C under 5.0% CO_2 atmospheric conditions for 48–72 h on tryptic soy agar (TSA) plates (Sigma A-22091) and/or leucine deficient medium (BMM) using established protocols [29, 30].

2.2. Construction of the gonadotropin expressing strains

To create the expression vectors, the *FliC* conjugated protein construct from Mizel, *et. al.* was utilized as a template [29, 30, 31]. The porcine *FSHβ* and *GnRH* epitope DNA sequences were obtained from NIH's NCBI Gene website, and the *FliC* DNA sequence was obtained from Verma, et. al. [30, 32]. FliC has been shown to be highly immunogenic [30]. *FliC-porcineFSHβ* and *FliC-GnRH* were codon optimized for *Brucella* species, synthesized and cloned into pUC, commercially, by GenScript (Piscataway, NJ). The commercially synthesized DNA sequences used are in the supplemental materials.

Restriction enzyme digests were run with BamHI and HindIII (Sigma Aldrich) to excise the construct from pUC, and cloned into the digested pNS4 plasmid following established protocols [26]. Our laboratory previously has demonstrated the use of this plasmid for antigen expression in *Brucella* [25, 26]. These ligated fragments were transformed into competent *E. coli* HB101. Each pNS4 construct was extracted following a QiaGen kit protocol and electroporated into competent strain RB51L following established protocols [25]. RB51L single colonies containing the pNS4 constructs grown on BMM were confirmed via polymerase chain reaction (PCR) with the following primer sets: FSHβ specific, 5′ CGCTGCAGTTTTGCTTCCTAT 3′ and 5′ CACTTGCCACAGTGACATTCG 3'; GnRH specific, 5′ TGGTCCTATGGCCTCCGT 3′ and 5′ TTATAGCTCCAATGTTCGCCGGA 3'. After PCR confirmation, Eton Biosciences (San Diego, CA) sequenced the extracted plasmids from strains RB51LFSHβ and RB51LGNRH for confirmation.

2.3. Preparation of protein extract and western blotting

Expression of the cloned antigens in the pNS4 plasmids was confirmed by western blotting using his-tag antibodies. Strains RB51L, RB51LFSHβ, and RB51LGnRH were grown in liquid BMM to mid-log phase and incubated in boiling water for 1 h then pelleted for 5 min in a microcentrifuge tube at 12,000 x g force. The pellets were washed twice with 10mM Tris-base (pH 8.1) and incubated at 60 °C for 20 min. The suspensions were combined with Laemmli-mercaptoethanol (1:1) buffer and heated in a water bath at 95 °C for 5 min. The suspensions were clarified of insoluble matter by centrifugation for 5 min at 12,000 x g. Samples were loaded into a 4–16% gradient Tris-Glycine protein gel (ThermoFisher Scientific) and run for 50 min at 90 V, and then transferred to Amersham Hybond P 0.45 PVDF 0.45 um 80 × 90 mm Blotting Membrane (GE Healthcare Life Science). Western blot analysis was performed on blotted proteins using a 1:3000 dilution of mouse IgG2a his-tag or porcineFSHβ horseradish peroxidase (HRP) conjugated primary antibodies (BioRad) overnight at 4 °C. Analysis was completed after adding 100uL of SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific) on the membrane and reactive bands were identified using Proteinsimple's FluorChem M.

2.4. Evaluation of clearance in BALB/c mice

Strain RB51L has been rigorously evaluated in mice previously for its ability to maintain expression plasmids without significant effects on growth characteristics in mice [25, 26]. The effects of the gonadotropin pNS4 plasmids were similarly characterized to ensure similar findings to Rajasekaran, *et al.* [25]. Three 4–6 weeks old female BALB/c mice per strain were vaccinated IP with 2×10^8 CFUs/mL of RB51LFSHβ or RB51LGnRH. One mouse each was euthanized at 4, 6, and 8 weeks post-vaccination. The spleens of each mouse were homogenized in 1mL of sterile saline under sterile conditions and serial dilutions plated on TSA. After 48–72 h of

incubation the colonies were counted and the Log_{10} CFUs per mL of homogenized spleen calculated.

2.5. Breeding study

The murine breeding study was designed based on a JAX industry monogamous pairing protocols [33, 34]. Power analysis, with 90% confidence intervals, determined 3 mice per group were needed to determine the effects of treatment on individual fertility characteristics. The study consisted of negative control groups (phosphate buffered saline (PBS) and strain RB51L) with 3 male and 3 female BALB/c mice each, a positive control group (Improvest®) with 3 male and 3 female BALB/c mice, and the gonadotropin expressing vaccine candidate groups (strains RB51LFSHβ and RB51LGnRH) with 5 male and 5 female BALB/c mice each for a total of 38 mice. The groups were vaccinated at Day 0 and boosted on Day 42, using a 22G 1inch needle, via either the intraperitoneal (IP) or subcutaneous route (SQ) with one of the following: PBS – 200uL IP; Improvest® (Lot#159600) – 50uL SQ; RB51L – 2.0×10^8 CFUs (2.0×10^7 CFUs for booster) IP; RB51LFSHβ - 2.0×10^8 CFUs (2.0×10^7 CFUs for booster) IP; and RB51LGnRH - 2.0×10^8 CFUs (2.0×10^7 CFUs for booster) IP. On Day 56 a handful of male bedding was added to the corresponding female cages to synchronize their estrous cycles. On Day 59 the males and females were randomly paired monogamously within each group. The females were observed daily for the presence of sperm plugs; if a plug was present this was considered gestation day E0.5. On Day 81, blood, body weight, testis weight, testis, epididymal sperm numbers/malformations, spleen, and liver samples were collected or recorded from male mice. On calculated day E15.5 (Day 86) of pregnancy, blood, body weight, uterine weight, uterus, fetal numbers/malformations, spleen, and liver samples were collected or recorded from female mice [35]. Blood was collected from anesthetized mice retro-orbitally, centrifuged, the serum collected, and then stored at -80 °C for future use in ELISAs. Spleen and liver samples were immediately stored at -80 °C for culturing, and the testis/uterus samples were stored in 10% neutral buffered formalin (Sigma Aldrich) for histopathology.

2.6. Sperm collection and evaluation

One testis of each male was placed in 500uL of 1% trisodium citrate solution and the epididymis macerated with #11 scalpel blades. After 2 min of incubation at room temperature, 10ul was added to each side of a hemocytometer. The sperm were counted and evaluated, with the groups blinded, following established protocols [23, 36].

2.7. Embryo and placental evaluation

Protocols from The Guide to Investigation of Mouse Pregnancy were used for the placental analysis [37]. After removal from the body cavity, the uterus was observed for embryo number with the groups blinded. Each embryo was then dissected and observed under the microscope for placental and embryo viability following protocols and descriptions [38, 39, 40, 41].

2.8. Histopathology

One testis from each male was placed in cassettes in 10% neutral buffered formalin (Sigma-Aldrich) for several weeks. Fixed testes were prepared by ViTALs histopathology lab at the VMCVM Veterinary Teaching Hospital. The hematoxylin and eosin (H&E) stained slides were randomly evaluated for the following: seminiferous tubule diameter of 20 random end-on tubules using an eye-piece micrometer at 40x after verification of scale with *Trichuris* eggs, spermatogonia and spermatid numbers, epididymal sperm concentrations, tubule structure, and signs of epididymitis and orchitis. A board certified pathologist confirmed these histopathologic findings.

2.9. Enzyme-linked immunosorbent assay (ELISA)

Testosterone concentrations were determined using a Cayman Chemical (Ann Arbor, MI) testosterone ELISA kit (#582701) following their kit protocols. The presence of GnRH and FSH antibodies was confirmed via absorbance values with the following protocol: Antigens (Provided by Boster and BEI Resources, VA, USA) were diluted in PBS to a concentration of 20 μg/ml. Wells of polystyrene plates (Nunc-Immunoplate with maxisorp surface) were coated with each antigen. Following a 2-h incubation at room temperature, plates were washed four times with a washing buffer (PBS at pH 7.4, 0.05% Tween 20) and then blocked with 2% bovine serum albumin (BSA) in PBS. After 1-hour incubation at 37 °C, plates were washed 4 times with 100 uL of wash buffer and then incubated with 50uL of the appropriate mouse serum. The plates were incubated for 4 h at room temperature and then washed four times with a washing buffer. Horseradish peroxidase-labeled goat anti-mouse IgG/IgG_2 (Southern Biotechnology, AL-USA) was added to the wells at 1:10,000 dilution. After 1 h of incubation at room temperature in the dark, the plates were washed four times with a washing buffer. One hundred microliters of substrate solution (TMB Microwell peroxidase substrate KPL, MD, USA) were applied to each well. After 20 min of incubation in the dark, the enzymatic reaction was stopped by adding 100 μl of stop solution (0.185 M sulfuric acid) and absorbance was measured at 450 nm using a microplate reader.

2.10. Ethics statement

All studies were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC) (#18–034), and followed biosafety level 3 standard operating procedures and protocols.

2.11. Statistical analysis

All statistical analysis was done using SAS Studio University Edition and Prism 8.4. Group and time-point data were compared using a One-Way ANOVA with Tukey's comparison test. Differences were considered to be significant when p values were less than 0.05. Strain RB51L and PBS treated groups were used as negative controls and Improvest® treated groups were used as a positive control. The diestral female's pregnancy data was removed from the strain RB51LFSHβ immunized group, as it's not possible for a non-cycling female to get pregnant. Binomial data was analyzed using odds ratios.

3. Results

3.1. Clearance, construction and confirmation of B. abortus RB51LFSHβ and B. abortus RB51LGnRH

The pNS4 plasmids containing DNA encoding FliC conjugated to gonadotropins were transformed into the strain RB51L. Transformed clones of strains RB51LFSHβ and RB51LGnRH were selected for stock cultures and confirmed to contain the pNS4-FliC-gonadotropin plasmids via PCR. Anti-his-tag western blotting of protein extract from strain RB51LGnRH culture revealed a his-tagged protein between 37 and 50 kDa (Figure 1). This is consistent with the estimated molecular weight of the FliC-GnRH protein (theoretically 39 kDa) plus the 6x His-tag (theoretically 1 kDa). Neither protein extracts from strains RB51L nor RB51LFSHβ culture showed any bands via anti-his-tag nor anti-porcine-FSHβ western blotting. Strain RB51LGnRH colonies recovered from inoculated mouse spleens at 28 days post-inoculation were confirmed to contain the pNS4-FliC-gonadotropin plasmid through PCR. Strain RB51LFSHβ colonies screened at 28 days or 42 days post inoculation did not contain the plasmid (Supplemental Figure 1AB). In BALB/c mice, strain RB51LGNRH was cleared from the mice by day 28 post-inoculation and strain RB51LFSHβ cleared by day 56 post-inoculation (Supplemental Figure 2).

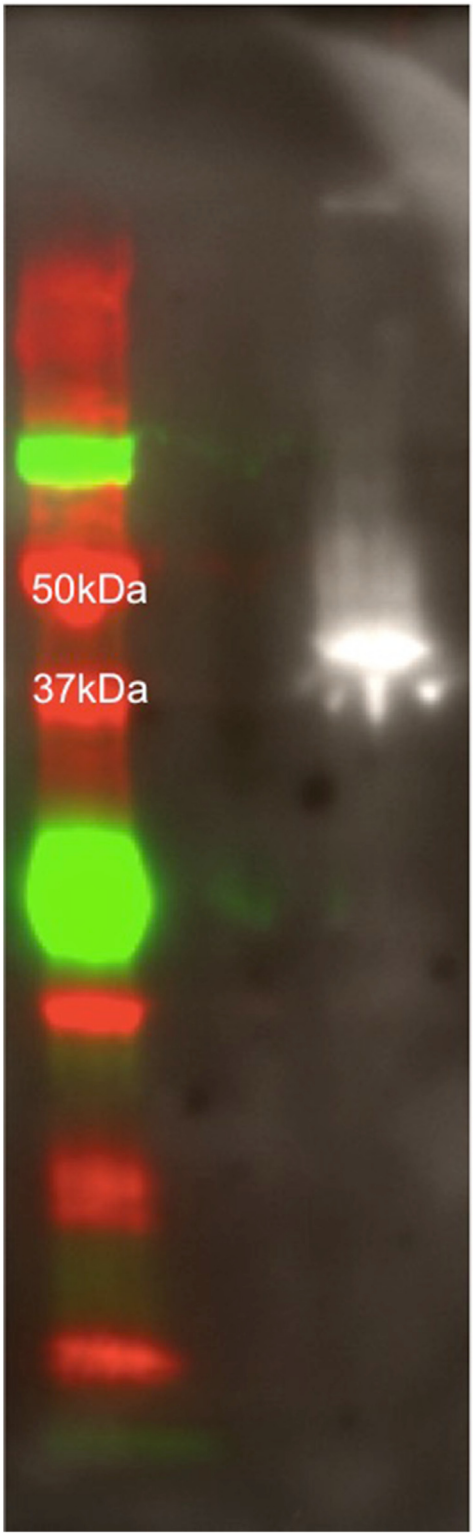

Figure 1. Anti-his-tag Western blot. Fluorescent anti-his-tag antibodies were applied to blotted protein extracts. Lane-1 consists of the BioRad Precision Plus Protein Ladder. Lane-2 consists of protein extract from strain RB51L, showing no significant bands. Lane-3 consists of protein extract from strain RB51LGnRH isolated from the spleens of BABLB/c mice 14 days post inoculation, with a his-tag protein between 37 kDa and 50 kDa. This corresponds with the theoretical size of the his-tagged FliC-GnRH protein (≈40kDa). The original pictograph is represented in Supplemental Figure 4.

3.2. Effects of B. abortus RB51LFSHβ and B. abortus RB51LGnRH on male BALB/c mice

The male mice were euthanized 39 days post-immunization and the carcasses of each group were weighed and found to have no statistical differences between them. After removal of the testes, their weight and volume were determined. The calculated testis volumes for the strain RB51LFSHβ immunized males (31.03mm^3) and strain RB51LGnRH immunized males (30.55mm^3) were statistically smaller than the control strain RB51L immunized males (51.13mm^3). The Improvest® immunized males (33.58mm^3) had statistically smaller calculated testis volumes compared to the PBS immunized control males (58.24mm^3). The average of ten to twenty random end-on seminiferous tubule diameters of the Improvest® immunized male group (85.03nm) were statistically lower than the PBS immunized male control group (90.77nm). The tubule diameters of the strain RB51LFSH immunized males (88.24nm) and strain RB51LGnRH immunized males (90.48nm) were statistically lower than the strain RB51L immunized male control group (95.47nm). The testosterone levels between all groups: PBS immunized males (3.5 pg/mL), strain RB51L (3.9 pg/mL), strain RB51LGnRH (3.7 pg/mL), and strain RB51LFSHβ (4.0 pg/mL) immunized males were statistically similar. These data points can be seen in Table 1 and Figure 2AB.

Ultimately, the males immunized with Improvest® (283,333 sperm/mL), strain RB51L (566,667 sperm/mL), strain RB51LFSHβ (250,000 sperm/mL), and strain RB51LGnRH (170,000 sperm/mL) had statistically lower sperm counts than those of the PBS (1,316,667 sperm/mL) immunized group (Figure 3). Their sperm motility and sperm morphology were observed but not quantitated. Despite differences in sperm concentration and testis values there were no overarching histological changes to the testicular structure between the groups (Supplemental Figure 3). This is most likely due to the high percentage of disruption in spermatogenesis needed to observe degeneration [42]. GnRH IgG ELISA OD_{450} values were statistically higher in the Improvest® (1.75) and strain RB51LGnRH (1.68) immunized grouped female plus male sera compared to the PBS (0.43) immunized grouped female plus male sera, but not to the strain RB51L (1.02) immunized grouped mouse sera (Figure 4A). PorcineFSHβ IgG ELISA OD_{450} values were statistically higher in the strain RB51LFSHβ (1.86) immunized grouped female and male sera compared to the PBS (0.806) and strain RB51L (1.04) immunized grouped female and male sera (Figure 4B). There were no organisms isolated from the spleens or livers from any male or female mouse in any of the breeding study groups.

3.3. Effects of B. abortus RB51LFSHβ and B. abortus RB51LGnRH on female BALB/c mice

The mice were monogamously paired on day 1 after estrous synchronization, and sperm plugs were noted on the mornings of days 2–4. Females were checked every morning for sperm plugs until euthanasia of the males. No other sperm plugs were noted (Table 2). The female carcass weights of the strain RB51LGnRH immunized group (26.67g) was statistically lower than the strain RB51L immunized group (32.18g). There were no statistical differences between the other group uterus weights (Table 2). There were statistically lower numbers of total fetuses in the mice immunized with strain RB51LFSHβ (Total: 7.2) and strain RB51LGnRH (Total: 9) compared to the strain RB51L immunized control group mice (Total: 11.3) (Table 2).

The odds ratios for the groups are as follows: mice vaccinated with strain RB51LFSHβ had 18.3 (95% CI 2.2–153.2) times the odds of having non-viable fetuses than mice vaccinated with strain RB51L or PBS, mice vaccinated with strain RB51LGnRH had 56 (95% CI 7.1–439.5) times the odds of having non-viable fetuses than mice vaccinated with strain RB51L or PBS, and mice vaccinated with a strain of RB51L expressing gonadotropins had 35.7 (95% CI 4.7–270.9) times the odds of having

Table 1. Male data.

Group	PBS	Improvest®	RB51L	RB51LFSHβ	RB51LGnRH
Carcass Weight	26.4 ± 1g	25.3 ± 2.6g	23.4 ± 1.1g	23.1 ± 2.6g	25.0 ± 1.9g
Testis Weight	73.0 ± 26.7mg	66.7 ± 31.3mg[a]	80.3 ± 22.6mg	51.6 ± 18.4mg[ab]	66.2 ± 5.8mg[a,b]
Testosterone Serum Levels	3.5 ± 0.02 pg/mL	3.4 ± 0.02 pg/mL	3.9 ± 0.06 pg/mL	4.0 ± 0.05 pg/mL	3.7 ± 0.07 pg/mL

This table shows the carcass weights in grams, testis weights in milligrams, and calculated testis volumes in millimeters cubed from the male mice post euthanasia from all of the groups. All averages are shown with 95% confidence intervals.

[a] indicates that these values were significantly lower than the PBS immunized group with the following *p* values: 0.01, 0.0003, and 0.006 respectively.

[b] indicates that these values were significantly lower than the strain RB51L immunized group with the following *p* values: 0.006 and 0.01 respectively.

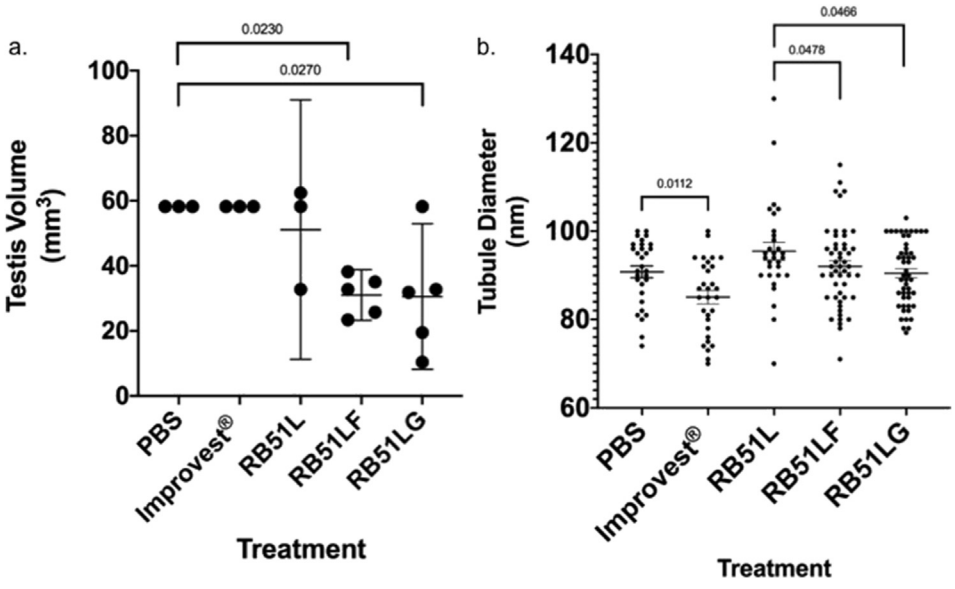

Figure 2. Testis volume and seminiferous tubule diameter in male BALB/c mice (a) The length, width, and height of the right formalin fixed testis of each male mouse was measured using calipers, 81 days post-vaccination. These measurements were used to calculate the testis volumes. The testis volumes for the strain RB51LFSHβ and RB51LGnRH immunized groups were significantly lower than the volumes from the males in the PBS group. The *p* values are as follows: 0.0230 and 0.0270. The significant differences between the testis volumes for the strain RB51LFSHβ and RB51LGnRH immunized groups were significantly lower than the volumes from the males in the Improvest® group in the same manner as the significant differences from the PBS group. (b) After 10% formalin fixation, the ViTals histopathology laboratory at the Virginia Tech Teaching Hospital mounted the right testis of each male and then hematoxylin and eosin stained them. At 40x magnification the micrometer was calibrated and end-on seminiferous tubules were randomly selected. Each tubule was measured from basal cell layer to basal cell layer. All group values were compared via One-way ANOVA, and the statistically significant differences are shown. The tubule diameters of the Improvest® group were statistically lower than the PBS control group, while the tubule diameters of the strain RB51LFSHβ and RB51LGnRH immunized groups were statistically lower than the strain RB51L immunized control group. The *p* values were as follows: 0.0112, 0.0476, and 0.0466. There were no significant differences between groups without brackets and *p* values.

non-viable fetuses than mice vaccinated with strain RB51L or PBS,.all of these values are significant. Mice vaccinated with Improvest® had 1.3 (95% CI 0.1–33.5) times the odds of having non-viable fetuses than mice vaccinated with strain RB51L or PBS, which was not significant. Upon gross and microscopic examination of each uterus and placenta, there was distention of the uterine artery in gravid uteruses. There was a lack of vascularity in the noticeably smaller fetus-placenta units from the strain RB51LFSHβ and strain RB51LGnRH immunized groups. Uteruses can be seen in Figure 5a-e, and examples of the microscopic evaluation of placental vascularity and fetal viability can be seen in Figure 6. There was a diestral uterus in the strain RB51LFSHβ immunized group (mouse #1). There were injection site reactions to the Improvest® SQ injections and mice had to be treated by the IACUC veterinarian. One female was euthanized due to the severity.

4. Discussion

The statistically lower average fetal numbers of strain RB51LFSHβ and RB51LGnRH immunized female mice demonstrate that the vaccine candidates affected fertility. This is difficult to achieve in the murine model, and will be discussed further towards the end of this discussion [42]. This effect is further supported with the strain RB51LFSHβ and RB51LGnRH immunized groups having lower uterine weights and higher odds ratios for non-viable fetuses, compared to the control groups. There are several limitations with this study that could affect this data, though. The males remained with the females until euthanasia making it a possibility that the smaller fetuses, classified as non-viable, in Figure 5a,c,d,e are viable fetuses. This is a feasible argument against the viability counts, but this does not impact the overall analysis of the impact on female fertility. The total fetal counts were statistically lower in strain RB51LFSHβ and RB51LGnRH immunized females. Furthermore, these findings could be further validated if the females were allowed to give birth for live pup counts, or if the males were removed after the presence of a sperm plug. However, this was beyond the ethical scope of this study. It was designed to mimic the natural reality of multiple copulations. Plus, the prediction of fertilization via the presence of a sperm plug is unreliable [37]. Nonetheless, the females were checked every morning for sperm plugs, and the only sperm plugs observed were within the first four days post pairing. This suggests that there were only copulations in the first four days. Another analytical limitation with this study is the fact

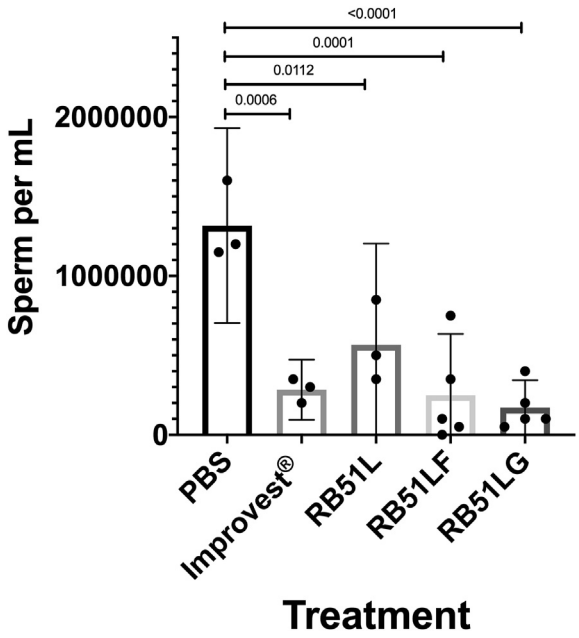

Figure 3. Sperm numbers per mL from male mice. Eighty-one days post vaccination, the epididymis of the left testicle was macerated and escaped sperm counted on a hemocytometer. After calculating the sperm per milliliter of solution, the values were analyzed with a one-way ANOVA. All group values were compared via One-way ANOVA, and the statistically significant differences are shown. The immunized groups Improvest®, RB51L, RB51LFSHβ, and RB51LGnRH had statistically lower sperm numbers per mL than the PBS control group. The *p* values were as follows: 0.0006, 0.0112, 0.0001, and <0.0001 respectively. There were no significant differences between groups without brackets and *p* values.

that the placenta can not be fully evaluated microscopically until estimated gestation day 7.5. Thus, uterine artery distension, fetal sizes, distribution of the fetuses, and the overall appearance of the uterus/-placenta were used to further characterize viability [37].

In strains RB51LFSHβ, RB51LGnRH, and Improvest® immunized males, the sperm concentrations, testis volumes, and testis weights were lower compared to the PBS control males, further supporting a vaccine effect. Testicular volumes and weights are good prognostic indicators of fertility, and the vaccinated males in this study prognostically had lower fertility compared to PBS control mice. The PBS control mice in this study had fertility characteristics that fall within the normal parameters of BALB/c male mice reported in the literature [14, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26].

The link between the vaccinations and these effects on the male gonad is further supported via the antibody ELISA absorbance and testosterone values. Following the hypothalamic-pituitary-gonadal axis, the reduced testosterone levels, sperm counts, seminiferous tubule diameters, and testis volumes of the strain RB51LGnRH immunized group suggest that the GnRH antibodies detected on ELISA are disrupting the entire pathway [14, 41]. In male mice immunized with strain RB51LFSHβ, the testosterone levels were more similar to the control mice. They also had reduced sperm counts, seminiferous tubule diameters, and testis volumes suggesting that the porcine FSHβ antibodies detected on ELISA were interrupting the hypothalamic-pituitary-gonadal axis at a lower level, as the production of testosterone was still being stimulated at a normal level. It is important to realize however, that testosterone is produced in waves. Thus, multiple serum samples over the length of the study would need to be analyzed to further verify this vaccine effect. It is also necessary to take into account that most mammals have circulating autoantibodies against their reproductive hormones [43, 44, 45, 46]. This could account for the ELISA absorbance values seen from the control mouse serum. Determination of the GnRH and FSHβ antibody ELISA absorbance values from pre-vaccinated serum would add validity to these conclusions. Determining the ELISA absorbance values two weeks after initial vaccination and booster vaccination would also provide a more complete data set, giving insight into the immunogenicity of these vaccine candidates. The number of anesthetized blood collections needed to address the above limitations was beyond the ethical scope of this study, though.

In a similar manner to the males, further testing of hormone levels and ovarian function would be needed to evaluate the extent of the

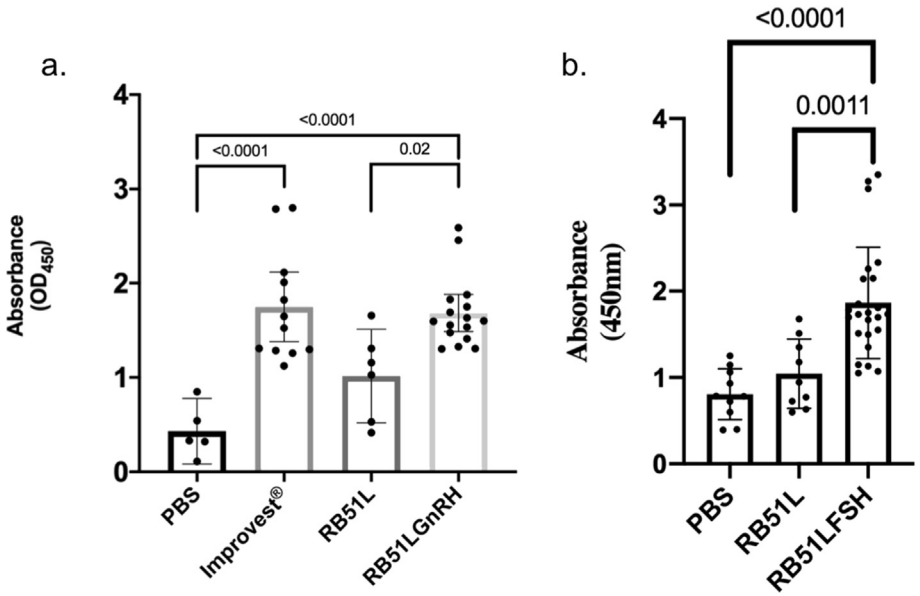

Figure 4. GnRH and porcineFSHβ IgG antibody ELISAs from male and female BALB/c mice. (a) Fifty microliters of serum collected 81 days post vaccination from both male and female mice were tested in duplicate for detection of anti-GnRH IgG antibodies. All group values were compared via One-way ANOVA, and the statistically significant differences are shown. The OD_{450} values from the serum of mice in the strain RB51LGnRH immunized group were significantly higher than those from the PBS control group with a *p* value of <0.0001. The OD_{450} values from the serum of mice in the Improvest® immunized group were significantly higher than those from the control group PBS, with the *p* value of <0.0001. The OD_{450} values from the serum of mice in the strain RB51LGnRH immunized group were significantly higher than those from the RB51L control group with a *p* value of 0.02. (b) Fifty microliters of serum collected 81 days post vaccination from both male and female mice were tested in duplicate for detection of porcineFSHβ IgG antibodies. All group values were compared via One-way ANOVA, and the statistically significant differences are shown. The OD_{450} values from the serum of mice in the strain RB51LF immunized group were significantly higher than those from the PBS control group with a *p* value of <0.0001 and the control RB51L group with a *p* value of 0.0011. This shows that there was an immune response amounted to the vaccines, which can be further seen by the other data sets. There were no significant differences between groups without brackets and *p* values.

Table 2. Female data.

Group	PBS	Improvest®	RB51L	RB51LFSHβ	RB51LGnRH
Sperm plug and date seen	3 of 3 on 1.3, 1.4, and 1.4.19	None seen	3 of 3 on 1.3, 1.3, and 1.4.19	4 of 5 on 1.3, 1.3, 1.3, and 1.4.19	3 of 5 on 1.2, 1.3, and 1.3.19
Carcass weight	32.89 ± 8.41g	34.91 ± 0.37g	32.18 ± 3.78g	30.43 ± 4.58g	26.67 ± 3.54g[a]
Uterus weight	7.51 ± 0.26g	9.49 ± 0.07g	4.75 ± 4.04g	3.46 ± 2.81g	2.98 ± 2.14g
Total Fetuses	10.3 ± 2.83 fetuses	8 ± 0 fetuses	11.3 ± 1.3 fetuses	7.2 ± 1.24 fetuses[b]	9 ± 1.38 fetuses[b]
Viable Fetuses	10 ± 2.26 fetuses	8 ± 0 fetuses	11.3 ± 1.3 fetuses	5.6 ± 2.77 fetuses[b]	4.8 ± 3.9 fetuses[b]
Non-viable Fetuses	0.3 ± 0.65 fetuses	0 fetuses	0 fetuses	1.6 ± 2.95 fetuses	4.2 ± 4.08 fetuses

This table shows the averaged carcass weights in grams, uterus weights in grams, and the number of sperm plugs seen and on what date they were seen per group from the female mice post euthanasia. The averaged total, viable, and non-viable fetus numbers per group are shown too. All averages are shown with 95% confidence intervals.

[a] indicates that these values were significantly lower than the RB51L group with the following *p* value 0.04.

[b] indicates that these values were significantly lower than the RB51L group with the following *p* values: 0.034, 0.035, 0.032, and 0.028 respectively from left to right across the chart.

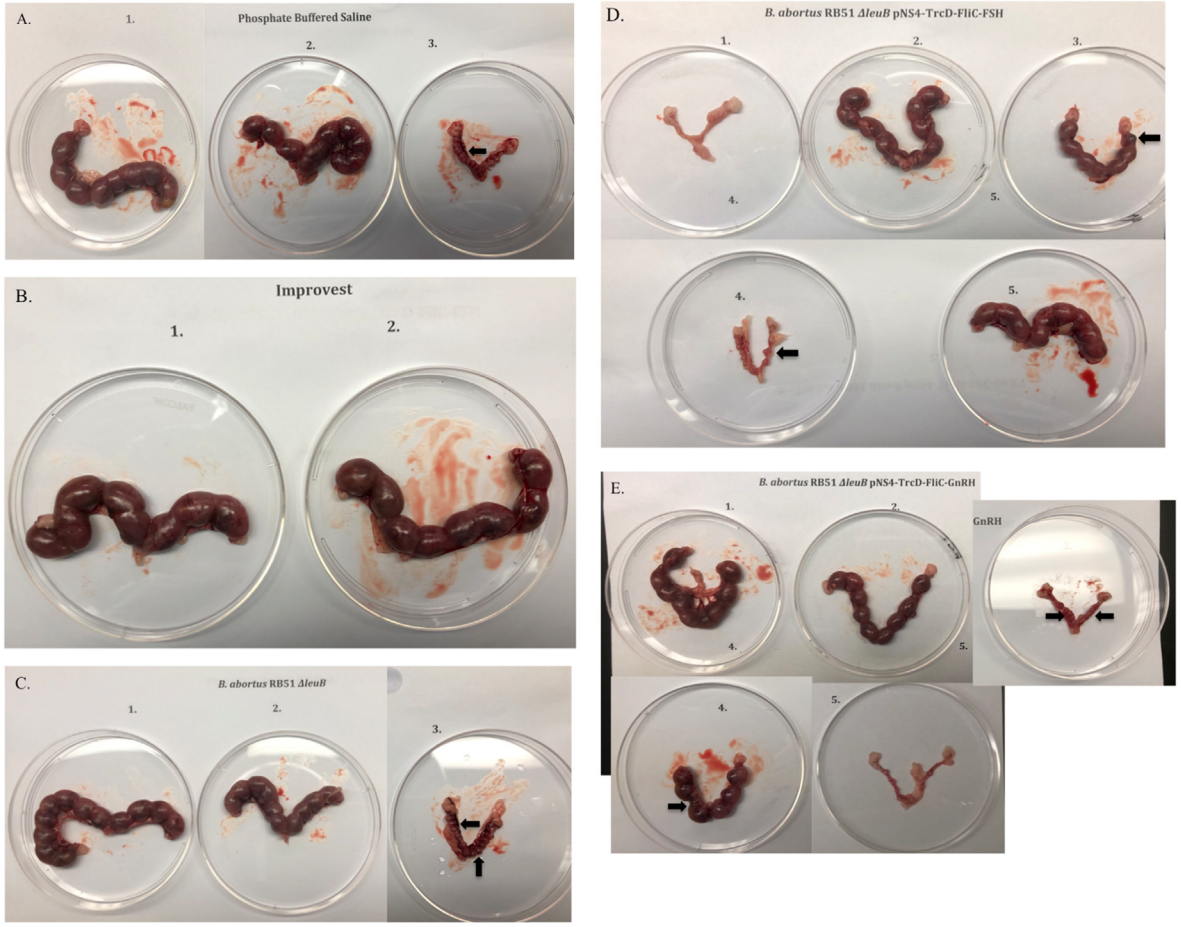

Figure 5. (a–e). Female gravid uteruses. One male and one female mouse per group were randomly paired after estrous synchronization 59 days after two vaccinations. On estimated day 15.5 of gestation the females were euthanized. All panels are described from left to right. (a) The PBS control group had 100% fertility and the uniform viable fetuses can be seen. Number 3 was clearly fertilized at minimum 7.5 days before euthanasia, as there were evaluable placentas. The arrow designates the distended uterine artery and dark placentas. (b) The Improvest® immunized group also had 100% fertility. (c) The control group immunized with strain RB51L had 100% fertility and the uniform viable fetuses can be seen. Number 3 was clearly fertilized at minimum 7.5 days before euthanasia, as there were evaluable placentas. The distended uterine artery and dark placentas are characteristic of viable fetuses and are designated by the arrows. (d) There were statistically fewer live and total fetus numbers compared to the strain RB51L immunized group and the strain RB51LFSHβ immunized group. Number 1 shows a diestral uterus, number 3 has an arrow pointing at a necrotic fetus, and number 4 has pale fetuses with abnormal distribution (designated by the arrow) and a lack of a noticeable uterine artery all characteristic of non-viable fetuses. (e) There were statistically fewer live and total fetus numbers compared to the strain RB51L immunized group and the strain RB51LGnRH immunized group. Number 3 has viable larger fetuses designated by the arrow on the left side and smaller pale fetuses with abnormal distribution designated by the arrow on the right side; there is a lack of a noticeable uterine artery all characteristic of non-viable fetuses. Number 4 has an arrow pointing at necrotic fetuses, which are clearly smaller than the viable fetuses. Finally, number 5 has small pale fetuses that are not uniform in size or distribution with a lack of a noticeable uterine artery, all characteristic of non-viable fetuses. The original pictograph is represented in Supplemental Figure 4.

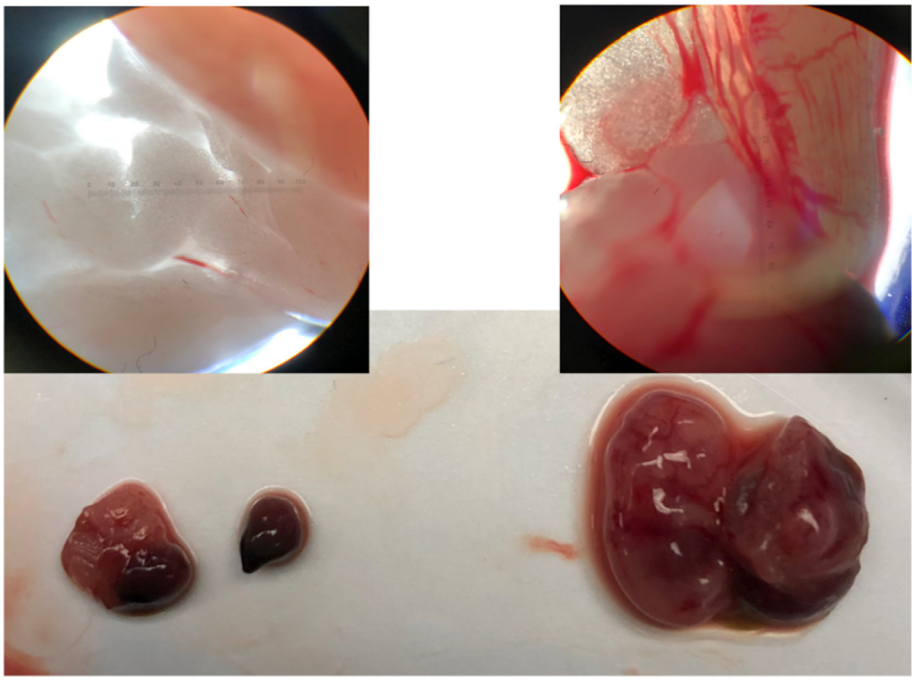

Figure 6. Microscopic evaluation of placental viability. All fetuses and placentas were evaluated following protocols and descriptions in Chapter 2 of The Guide to Investigation of Mouse Pregnancy. On estimated day 15.5 of gestation the females were euthanized and the fetal-placental units evaluated. On the left hand side of the figure a non-viable fetal-placental unit is shown. The lack of vascularity at 40X magnification along with the lack of form is characteristic of a non-viable fetus. FSH has been shown to have an effect on vascularity. On the right hand side of the figure a viable fetal-placental unit is shown. The vascularity at 40X magnification along with the recognizable form of the fetus is characteristic of a viable fetus. Represented by the strain RB51LGnRH immunized group.

vaccinations' effects on the strain RB51LFSHβ immunized diestral female. This finding could indicate a target level for porcine FSHβ IgG antibody ELISA absorbance values in female mice needed to produce 100% infertility. Ultimately this could suggest the need for a higher antibody response with an adjuvant, as the sera from diestral female had the highest FSHβ ELISA absorbance values [47].

The uniform robust antibody response needed to further inhibit fertility may be inhibited by the fact that FSH has been shown to have low expression due to a hairpin structure in its mRNA [43, 44]. This would reduce the amount of the protein produced by the vaccine strain, thereby causing a low immune response [48,49]. The protein has also been shown, in *E. coli,* to be expressed in inclusion bodies, which if true in *Brucella* could prevent a robust immune response. This could also reduce the likelihood of detection on Western blot [43, 44]. We did notice a decreased growth rate and lighter plasmid PCR bands (Supplemental Figure 1B) with the strain RB51LFSHβ compared to the other strains. Further testing would be needed to confirm this. In addition, further protocols for solubilization of protein inclusion bodies would need to be performed to further investigate the protein expression. Although, a FliC-porcineFSHβ protein was not explicitly detected through western blotting, the antibody ELISA data, fetal numbers, sperm concentrations, testis volumes, and other reduced fertility characteristics show that the vaccine candidates did have an effect compared to PBS control mice. This suggests that the strain RB51LFSHβ is producing a FliC-porcineFSHβ protein, *in vivo,* to produce an antibody response, all-be-it too low to confer 100% infertility.

A limitation in the use of FSH as an immunogenic target was identified in the literature, but expression and immune response studies would need to be done to confirm this in rodents and swine. It has been stated in literature that vaccination of humans with FSH does not reduce sperm concentrations to a low enough level to cause infertility, despite antibody responses [50]. These claims and observations could possibly be overcome through immunization with both vaccine strains, the use of FSHβ epitopes as a target, or even with the use of the USDA's AdjuVac™ adjuvant. This strong adjuvant has been shown to be effective for use with contraceptive vaccines in wildlife to stimulate robust immune responses [47]. The use of this adjuvant may also allow for a single injection of the vaccine candidates to provide immunocontraception, but this needs to be confirmed.

Although our vaccines did not induce 100% infertility, there are several important limitations to further consider. First, in regards to the strain RB5LFSHβ immunized group, the FSHβ subunit sequence used is specific for swine and not mice. Thus, further studies are needed in swine. Second, the use of mice was a major limitation in this study. Russell, *et al.* explicitly describes limitations to using rodents for fertility and breeding studies. These limitations include that spermatogenesis must be decreased by 90% or more to affect the number of progeny, that a vast numbers of animals is needed in a true breeding study, that there is a massive cost associated with an effective study, that rodents have the ability to store epididymal sperm, that male fertility is not FSH dependent, that rodents have seasonality to their estrus cycles, and that there are many uncontrollable variables that affect fertility [42, 51]. Some of these limitations could be over come by using purpose-bred swine. Other limitations to this study center around the groups not being maintained in the same room and not having the same number of mice. Even with a larger *n* value there would be wide variation in individual response to the vaccines, which can cause a skewed statistical analysis. Thus, it is important to analyze the biological significance of reduced fetal numbers, sperm concentrations, testes volumes, etc. This also highlights the limitations presented by the sparse blood samples taken for comparative analysis. All of these limitations point to the difficulty of breeding studies and the need for planned experiments in the target species (feral swine in the field).

Before field application several aspects of the vaccine candidates need to be experimentally determined. Some of those characteristics include vaccine shedding, the ability of antibodies or the vaccine to cause infertility to meat consumers, the vaccines' ability to prevent the spread of brucellosis in the field (our laboratories have previously published clearance and protection profiles of the leucine auxotrophic strain of RB51L [25, 26]), and oral vaccination efficacy. *Brucella* spp. naturally infect hosts via the oral route and therefore theoretically these vaccine candidates can be used as an oral vaccine [52, 53]. An oral vaccine bait would be easier and less expensive to produce/deploy in the field, although there are many hurdles to over come [53]. It would also be important to determine if these vaccine candidates combined with each other (strain RB51LFSHβ and RB51LGnRH), hunting, baiting, and/or trapping protocols could reduce feral swine reproduction enough to

maintain or decrease the current population. A combination approach seems to be necessary as there is significant literature evidence for the lack of developing a 100% effective feral swine immunocontraceptive vaccine [50].

These vaccine candidates could contribute to the effort to control feral swine as mice immunized with strain RB51LGnRH had antibody absorbance values statistically similar to the control mice vaccinated with the Zoetis vaccine, Improvest®. Improvest® has been used effectively worldwide for decades now [54]. The similarity is also seen in reduced testicular weight, reduced seminiferous tubule diameter, reduced reproduction, and having little to no effect on body weight compared to the controls seen in our data and the Improvest® literature [55, 56, 57, 58, 59]. It is important to remember that the Improvest® label claim states that it suppresses testicular function with no indication for inducing infertility in swine, and it has no indications for mice [11].

In conclusion, murine sperm production was not reduced by 100% and fertilization did occur, but the vaccines affected the dynamic and essential biological process of reproduction. There are limitations to this study including the murine model, the number of animals, the number of blood draws, antibody and hormone analysis, succinct histopathology analysis, and the fact that the FSHβ subunit is swine specific. Despite these limitations, these efforts demonstrate a reduction in fertility characteristics in both males and females, comparable to Improvest® in the literature. Ultimately, the vaccine candidates, *B. abortus* RB51LFSHβ and *B. abortus* RB51LGnRH, could provide a more cost-effective oral dual purpose immunocontraceptive brucellosis wildlife vaccine and should be further evaluated as an oral bait in feral swine.

Declarations

Author contribution statement

Steven G. Waldrop: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Garrett P. Smith, Stephen M. Boyle: Analyzed and interpreted the data; Wrote the paper.

Nammalwar Sriranganathan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by Smithfield Foods Murphy-Brown, LLC (Virginia Tech Foundation, Inc. Fund #444115), and the Virginia Maryland College of Veterinary Medicine's Office of Research and Graduate Studies.

Data availability statement

Data associated with this study has been deposited at VTechData https://data.lib.vt.edu/catalog?utf8=✓&q=stevenw3.

Competing interest statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2021.e06149.

Acknowledgements

We would like to thank the VMCVM's board certified pathologist, Dr. D. Phillip Sponenberg, for his histopathology expertise. We thank Dr. Stephen Werre and the Virginia Tech Statistical services for mentorship in statistical analysis. We thank Amanda Kravitz for her help in collecting samples, and Nancy Tenpenny's technical laboratory support.

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