

# Inherent Food Safety of a Synthetic Gonadotropin-Releasing Factor (GnRF) Vaccine for the Control of Boar Taint in Entire Male Pigs

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## ABSTRACT

Compared to compounds with a pharmacological mode of action, where the possible presence of drug residues in food is a public health concern, vaccines are generally considered safe from a food quality perspective. This is due to the intrinsic lability of these complex biological molecules, both in the body of the vaccinated animal and, if they should ever get so far, in the cooking process and/or intestinal tract of a consumer. The inherent food safety of a novel gonadotropin-releasing factor (GnRF) vaccine, intended to be administered by injection to male pigs for the control of boar taint, was confirmed using several animal models. In addition to conventional oral bioavailability studies, an experiment was also performed

to check for the presence of a direct hormonal effect of the vaccine antigen. The vaccine antigen comprises a synthetic analogue of mammalian GnRF covalently coupled to a carrier protein. Intravenous administration of this antigen in sheep had no effect on luteinizing hormone secretion from the pituitary gland, demonstrating that the vaccine itself has no hormonal activity. Repeated oral dosing of the vaccine to pigs failed to stimulate production of detectable circulating antibodies against GnRF and did not affect serum testosterone levels. This lack of oral activity was further confirmed by the oral administration to laboratory rats of graduated doses of the vaccine up to 70 times (on a weight for weight basis) the recommended injectable dose in pigs. There were no quantifiable vaccine antigen levels or anti-GnRF antibodies detectable in the sera of these rats at any dosage level, or any secondary effects on sex hormone levels, in-

dicating that vaccine given orally is neither systemically bioavailable nor immunogenic. These studies confirm that there is no risk to human health from the consumption of pork from pigs administered this boar taint vaccine.

## INTRODUCTION

Boar taint, caused principally by accumulation of androstenone and skatole in fatty tissue, is a significant food quality problem in sexually maturing male pigs. Androstenone is a pheromone steroid produced in the testes, and skatole is a by-product of the bacterial degradation of tryptophan in the large intestine. Both substances are highly lipophilic and are sequestered in the adipose tissue of the pig. Due to relatively high volatility, both compounds are readily released upon heating and cooking of pork and can give rise to an offensive odour (boar taint).<sup>1-4</sup>

There are 2 traditional management approaches to this meat quality problem: slaughter prior to sexual maturity and, much more commonly, surgical castration prior to weaning. Both practices have significant drawbacks however. Slaughter of pigs at relatively light weights results in significant production losses. Castration of very young pigs prevents endogenous production of male steroids that give rise to androstenone and skatole accumulation but causes increased fat deposition in the carcass, less lean meat yield, and statistically significant reductions in growth efficiency.<sup>5,6</sup> Because of poorer feed conversion efficiency, castrated pigs are significantly more expensive to raise than intact pigs. Aside from direct production losses, castration is also associated with increased mortality from post-castration complications such as infections and hernias. Anecdotally, this increase in mortality can be as high as 0.5 to 1.5 percentage points. Additionally, castration is criticised by animal welfare groups because it is generally practised without anaesthesia and is associated with pain-related behavior<sup>7</sup> and significant increases in serum cortisol concentrations indicative of stress.<sup>4,8</sup>

The economic and welfare drawbacks of surgical castration prompted the development of a parenteral vaccine (Improvac<sup>®</sup>/Vivax<sup>®</sup>; Pfizer Animal Health) that stimulates neutralizing antibodies directed against endogenous gonadotropin-releasing factor (GnRF).<sup>6,9</sup> Endogenous GnRF stimulates the pituitary-gonadal axis, which, in the boar, results in the synthesis of testicular steroids, including testosterone and androstenone. Suppressing testicular steroid synthesis not only prevents androstenone production but also accelerates hepatic clearance of skatole.<sup>4,10</sup> Thus, the net effect of inducing antibodies against circulating GnRF is the inhibition of testicular function and the consistent reduction of both androstenone and skatole to levels below consumer detection.

The immunizing antigen in the commercial vaccine comprises a synthetic analogue of endogenous mammalian GnRF conjugated to a carrier protein. Instead of having 10 amino acids like endogenous GnRF, the synthetic GnRF peptide lacks 1 amino acid and is thus foreign to the GnRF gonadotrope receptors in the pituitary gland. Covalent linkage of the GnRF analogue to the carrier protein results in an antigen that is even more foreign to the pituitary GnRF receptors but, together with the aqueous adjuvant in the vaccine formulation, allows stimulation of the immune system to transiently produce high levels of circulating antibodies to GnRF.

The studies described in this paper were conducted, firstly, to determine if the synthetic GnRF analogue or the vaccine antigen have any hormonal activity (Study 1) and, secondly, to see if orally administered vaccine antigen is systemically bioavailable or immunogenic (Studies 2 and 3). Negative results would confirm that there are no immunologic or endocrinologic safety hazards for humans consuming meat from vaccinated pigs.

## MATERIALS AND METHODS

All experiments involving animals were carried out in compliance with national legislation and subject to local ethical review.

## **Study 1. Evaluation of Hormone Activity of the GnRF Analogue and Antigen Conjugate**

A controlled experiment was performed to determine if either the GnRF analogue or the protein conjugate (vaccine antigen) have any direct hormonal activity when administered parenterally. As GnRF is highly conserved across mammalian species,<sup>11</sup> and because of extensive experience with a sheep model, the sheep was used as the test animal.

Twelve post-pubertal female crossbred sheep were randomly assigned to 1 of 4 groups (n = 3 each). The jugular vein was cannulated for blood sampling and intravenous (IV) injection of the test articles. On Day 8 of their respective oestrus cycles, when luteinizing hormone (LH) pulse frequency was low, sheep were given 3 IV injections of 20 mg of morphine at half-hour intervals to suppress synthesis of endogenous GnRF.<sup>12</sup> After the third morphine injection, the respective test groups were given either a single IV injection of saline, natural GnRF peptide (1 µg), synthetic GnRF peptide analogue (50 µg), or sufficient vaccine antigen to provide the equivalent amount of 50 µg of covalently bound GnRF peptide analogue. The 50-fold larger dose of GnRF peptide analogue compared with natural GnRF was estimated from the sequence of the analogue peptide and potency comparisons with other characterized peptide analogues. Baseline blood samples were obtained prior to treatment and at 10 intervals up to 240 minutes after injection (Figure 1). Plasma concentrations of LH were assayed using a standard radioimmunoassay previously described<sup>13</sup> with a detection limit of 0.11 ng/mL.

## **Study 2. Systemic Effects of Oral Administration of Vaccine in Pigs**

To evaluate the effects of oral ingestion of the vaccine, a controlled experiment was performed to determine the antibody and hormonal response in pigs following multiple vaccine doses given orally.

Pigs were chosen as the test animal for this study since the gastrointestinal tract of the pig is similar to that of humans and their

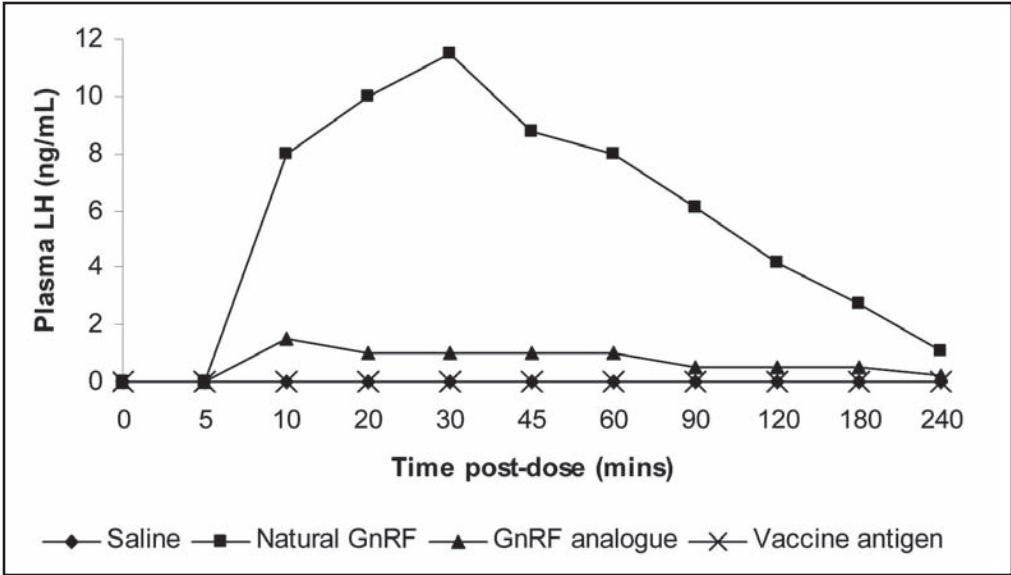
size allows simple administration of a full dose of the vaccine.

Twelve 12- to 13-week-old male pigs were randomly assigned to a treatment group or untreated control group (n = 6 each). Commercial vaccine was given to the treated group as a 2-mL oral dose by mixing with a small amount of pelleted feed, prior to normal feeding. This was similarly followed by a second oral dose 28 days later. Blood samples were obtained at 14, 28 and 42 days after the first oral treatment and assayed for serum testosterone and antibodies against GnRF. The sample taken 28 days after the first dose was obtained just prior to administration of the second dose. The sample taken 42 days after the first dose was obtained 14 days after the second dose: an interval that would normally allow an anamnestic immune response to be detected if it occurred. Samples were taken between 10 AM and noon to minimize diurnal variation in testosterone concentrations. Serum testosterone was measured using a commercial radioimmunoassay kit (Direct Testosterone Kit, Cat No. 135; Pantex, Santa Monica, CA, USA), expressed as ng/mL, and analysis of variance (ANOVA) used to compare results. Titers of antibody against GnRF were measured by a validated in-house radioimmunoassay, with titers expressed as reciprocals of the dilution that bound 30% of a commercial tritium-labeled GnRF tracer available from Amersham Inc (Piscataway, NJ, USA). Separation of bound from free GnRF was achieved with precipitation using bovine gamma globulin and 18% polyethylene glycol. The limit of quantification (LOQ) of this assay was 20 titer units.

## **Study 3. Oral Bioavailability and Systemic Effects of Vaccine in Rats**

A controlled study was conducted to evaluate systemic bioavailability, immunogenicity, and any indirect hormonal effects of the vaccine following oral and parenteral administration to Sprague-Dawley rats (a well-characterized laboratory animal model routinely used in toxicology studies). Table 1 summarizes the test groups, vaccine dos-

**Figure 1.** Mean plasma luteinizing-hormone (LH) concentrations following IV injection of sheep with either saline, 1 µg natural gonadotropin releasing factor (GnRF), 50 µg synthetic GnRF analogue, or vaccine conjugate antigen (sufficient to provide the equivalent amount of 50 µg of covalently bound GnRF peptide analogue). Limit of LH quantification = 0.11 ng/mL.



ages, and toxicological/immunological and other outcome parameters. Serum anti-GnRF antibody levels and systemic bioavailability of the antigen were determined by electrochemiluminescent immunoassays (ECLIA) with an LOQ of 4.7 pmol/mL for anti-GnRF antibodies and 1.4 pmol/mL or 0.098 µg/mL for vaccine antigen.

Commercial vaccine was administered orally by gavage once on Day 1 and on Day 29 to 5/sex/group male and female Sprague-Dawley rats at doses of 11.4 µg/kg, 272 µg/kg, and 462 µg/kg. These doses represented approximations of 1.7×, 41.2×, and 70×, respectively, of hypothetical oral consumption of a full 2-mL vaccine dose by a 60-kg human. An additional 2 groups of 5 males and 5 females were given either saline or formulation vehicle at the same dosing volume and dosing interval. A positive control group was also included that consisted of 5 males and 5 females given 27.5 µg/kg subcutaneously also on Day 1 and Day 29. Parameters for evaluation included daily clinical observations, weekly body weight, weekly food consumption, and terminal hematology, coagulation, clinical chemistry, and hormone analysis. Hormone analysis

included LH (all animals), progesterone (females only), estradiol-17β (females only), and testosterone (males only). Rats in the oral groups and in the SC injection positive control groups (Table 1) were necropsied on Day 58 of the study (ie, 29 days after the second dose). Organ weights on heart, liver, kidneys, adrenal glands, pituitary, and brain were obtained and representative sections of 47 tissues were collected for histological evaluation. Hormone data were analyzed using a mixed model ANOVA.

Systemic exposure and antibody response were assessed in toxicokinetic (TK) satellite groups in which 3/sex/group were dosed with 462 and 27.5 µg/kg for the high-dose oral dose and positive control SC dose groups, respectively, on Day 1 and Day 29. Blood samples were taken at 0 (pre-treatment), 1, 4, 8, 12, and 24 hours and 2, 7, 14, and 21 days after the first dose and at 0 (pre-treatment), 1, 4, 8, 12, and 24 hours and 2, 7, 14, and 21 days after the second dose from all animals in both TK treatment groups. The blood samples were processed into serum and were assayed for the vaccine antigen and anti-GnRF antibodies. The vaccine antigen and logarithm transformed anti-GnRF

**Table 1.** Rat Bioavailability Study Test Groups.

Test Group	Antigen Dose	Dosing Regimen	Toxicity Parameters
Sterile water control (n = 5/sex)	0 µg/kg	PO, day 1 and 29	1, 2
Vehicle control (n = 5/sex)	0 µg/kg	PO, day 1 and 29	1, 2
Oral-toxicity low dose (n = 5/sex)	11.4 µg/kg	PO, day 1 and 29	1, 2
Oral-toxicity mid dose (n = 5/sex)	272 µg/kg	PO, day 1 and 29	1, 2
Oral-toxicity high dose (n = 5/sex)	462 µg/kg	PO, day 1 and 29	1, 2
Positive control (n = 5/sex)	27.5 mg/kg	SC, day 1 and 29	1, 2
TK (n = 3/sex)	462 mg/kg	PO, day 1 and 29	2, 3, 4
TK positive control (n = 3/sex)	27.5 mg/kg	SC, day 1 and 29	2, 3, 4

PO = per os; SC = subcutaneous injection; TK = toxicokinetic.

Toxicity parameter key: (1) mortality, food consumption, body weight, terminal hematology and coagulation, clinical chemistry, gross necropsy, Day 58 histopathology; (2) hormone response; (3) anti-GnRF antibody; (4) GnRF analogue protein conjugate bioavailability.

values were analyzed with a mixed model ANOVA for repeated measures.

## RESULTS

### Study 1. Evaluation of Hormone Activity of the GnRF Analogue and Antigen Conjugate

The mean temporal responses for the 4 treatment groups are shown in Figure 1. Luteinizing hormone was not detectable in any of the 12 sheep prior to administration of their respective treatments, indicating that morphine effectively blocked LH secretion. All 3 sheep given 1 µg of natural GnRF had a rapid, marked increase in LH with a mean peak value of  $11.63 \pm 5.03$  ng/mL. The 3 sheep given unconjugated GnRF peptide analogue at a dosage 50 times greater than

that for natural GnRF had a relatively small increase in LH, with a mean value approximately 10-fold less than that for sheep given natural GnRF at a 50 times lower dose. Sheep given either the GnRF analogue-protein conjugate (vaccine antigen) or saline produced no quantifiable LH response. The test determined that the GnRF peptide analogue had a relative activity of only 0.2% compared to natural GnRF (mean GnRF analogue response  $\div$  mean GnRF response  $\div$  50  $\times$  100%), while the vaccine conjugate antigen had no LH stimulating activity.

### Study 2. Systemic Effects of Oral Administration of Vaccine in Pigs

The serum anti-GnRF antibody titer for all samples was <20, the minimum detectable level, following both the first and second oral vaccine doses. Mean serum testosterone levels at the 3 sampling intervals are shown in Table 2. All pigs had measurable testosterone levels that were within normal reference ranges for animals of that age. There were no significant differences in testosterone levels between orally dosed pigs and untreated control pigs at any sampling interval. Throughout the trial, daily observations of the test animals revealed no abnormal clinical signs or adverse events in any of the pigs.

**Table 2.** Mean Serum Testosterone Concentration in Pigs Given Oral GnRF Analogue Protein Conjugate Vaccine.

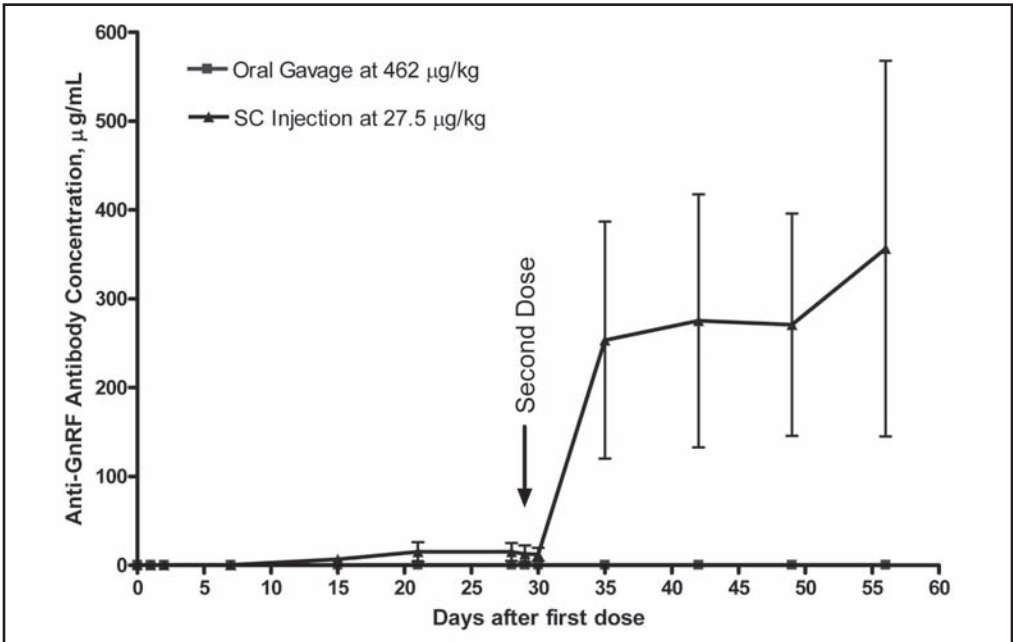
Test Group (n = 6 each)	Mean Serum Testosterone (ng/mL) $\pm$ SD		
	Day 14	Day 28	Day 42
Untreated controls	0.69 ( $\pm$ 0.35)	1.04 ( $\pm$ 0.35)	1.18 ( $\pm$ 0.49)
Vaccine treated	1.33 ( $\pm$ 0.84)	1.15 ( $\pm$ 0.49)	0.92 ( $\pm$ 0.26)
P-value	0.44	0.59	0.57

### Study 3. Oral Bioavailability and Systemic Effects of Vaccine in Rats

All vaccine dose levels and routes of administration were clinically well tolerated. All rats



**Figure 2.** Antibody response after oral and subcutaneous administration of vaccine in rats.



survived to termination of the study and there were no significant differences in body weight or food consumption. Post-treatment haematology, coagulation, and clinical chemistry values were not affected by treatment. Occasional individual variations in these parameters were random, generally small, and were not associated with vaccination route, dosage size, or other parameters or with histological findings. Orally treated rats had no test article-related histopathological effects. There were no quantifiable anti-GnRF antibody responses in any of the rats in the TK group given vaccine orally. There were also no quantifiable antigen levels in the serum of any rat in the TK groups at any of the sampling times, whether the vaccine was administered orally or by SC injection. Unfortunately, because of instability of the GnRF conjugate in frozen serum, the effective LOQ of the assay was estimated to be ~1 µg/mL rather than the 0.098 µg/mL determined during method validation.

As expected, parenterally vaccinated (positive control) rats had significant anti-GnRF antibody responses as compared to the orally treated rats (Figure 2,  $P = 0.033$ ) and significant decreases in serum hor-

mones, testosterone in male and progesterone in female rats (Table 3) relative to the control and the orally treated rats. Serum levels of LH and estradiol-17β in the parenterally vaccinated rats were not significantly different from the serum levels of these hormones in the control and orally treated rats. At necropsy, the parenterally vaccinated rats had undersized sex glands/reproductive organs, ie, testes, seminal vesicles, prostate glands, or uteri.

Based on these results, an oral no-observed-effect level (NOEL) for the GnRF analogue conjugate (vaccine antigen) was considered to be 462 µg/kg, the highest oral dosage level given. On a weight-for-weight basis, this oral NOEL is approximately 70-fold greater than the recommended 2 mL injectable vaccine dose in pigs.

## DISCUSSION

Intravenous administration to sheep of the GnRF conjugate antigen used in the commercial vaccine demonstrated that this antigen has no innate hormonal activity. The minor LH-stimulating effect of the unconjugated GnRF analogue (approximately 0.2% the effect of natural GnRF) was completely

**Table 3.** Rat Bioavailability Study Mean Serum Hormone Assay Results.

Test Group	Antigen Dose (Route)	Testosterone (Male) ng/mL	Progesterone (Female) ng/mL
Sterile water control	0 µg/kg (PO)	76.0 ± 27.0	6.10 ± 1.65
Vehicle control	0 µg/kg (PO)	67.3 ± 23.9	8.67 ± 2.24
Oral-toxicity low dose	11.4 µg/kg (PO)	74.2 ± 26.4	10.3 ± 2.63
Oral-toxicity mid dose	272 µg/kg (PO)	103 ± 36.5	11.4 ± 2.88
Oral-toxicity high dose	462 µg/kg (PO)	53.3 ± 19.1	7.20 ± 1.90
Positive control	27.5 mg/kg (SC)	13.5 ± 5.1*	2.34 ± 0.77*
TK high dose	462 mg/kg (PO)	120 ± 40.1	5.00 ± 1.34
TK positive control	27.5 mg/kg (SC)	16.2 ± 5.7†	1.45 ± 0.55†

PO = per os; SC = subcutaneous injection; TK = toxicokinetic.

\**P* < 0.05 vs all other main study treatment groups.

†*P* < 0.05 vs oral TK treatment group.

eliminated after conjugation with the carrier protein. This experiment was designed to test an extreme challenge in the test animal, by direct injection into the blood stream of a high dose of peptide, and of the equivalent amount of peptide presented as conjugate. The complete lack of hormonal activity of the antigen provides compelling evidence that no direct hormonal effect could occur from the hypothetical human consumption of antigen in the meat from a vaccinated animal.

Pigs that were administered the vaccine orally had no detectable antibody response or interference with normal testosterone levels (Table 2). As may be expected with a protein, these results demonstrate an absence of bioavailability following oral ingestion of the GnRF analogue-protein conjugate (vaccine antigen). Negative serum antibody results 14 days after the second oral vaccine dose were noteworthy because an anamnestic response would have occurred within that time period if oral administration were capable of eliciting a systemic immune response. In terms of human food safety, this feeding experiment, which was designed to be sensitive for the detection of an immune response, provides strong evidence that hypothetical human consumption of vaccine residues would not induce antibodies to GnRF or have any secondary endocrinological effect.

As evidenced by the contrasting se-

rologic response of laboratory rats given the vaccine orally or by SC injection, an immune response occurs only when the vaccine is given by injection (Figure 2). Oral administration of the vaccine to rats failed to stimulate anti-GnRF antibodies, corroborating the results of the pig oral administration experiment. Furthermore, oral administration was toxicologically innocuous even when vaccine was given at a relative dose of 70 times that recommended by SC injection for pigs. Administration of vaccine by SC injection had no toxicological effect in rats, either clinically or by objective biochemical parameters. Quantifiable levels of the antigen (LOQ = 1 µg/mL) could not be found in any of the rats in either the orally dosed groups or the subcutaneously dosed group. In summary, the experiments in laboratory animals indicate that the vaccine administered orally is neither toxic nor systemically available nor immunogenic.

As far as we are aware, oral absorption of active residues in meat from any protein subunit vaccine given parenterally to food-producing animals has never been demonstrated. Given the protein composition of vaccine antigens, expected rapid metabolism in the animal host after injection, the fact that slaughter almost always occurs weeks or even months after vaccination, and that meat is usually cooked prior to eating, consumption of intact vaccine antigens is highly unlikely. Nevertheless, consumer insistence

on food safety for any product that is used in livestock is rightfully placed. These considerations justify the safety studies described in this report. The results, demonstrating that the antigen in this boar taint vaccine has no intrinsic hormonal activity and is neither systemically available nor immunogenic by the oral route, affirm the food safety of a product concept that has been safely and effectively used in the field for nearly a decade.<sup>14,15</sup>

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