Revised

Serum antibody immunoreactivity and safety of native porcine and recombinant zona pellucida vaccines formulated with a non-Freund's adjuvant in horses

Margaret B. Nolan[a,b], Martin L. Schulman[a,b] , Alma E Botha[a], Anne-Marie Human[b], Robyn Roth[c], Michael C Crampton[c], Henk J. Bertschinger[a,b]

[a] Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

[b] Veterinary Population Management Laboratory, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

[c] Biomanufacturing Technologies, Biosciences, Council for Scientific and Industrial Research, Pretoria, South Africa

martin.schulman@up.ac.za; ester.botha@up.ac.za; annemarie.human@up.ac.za; rroth@csir.co.za; mcrampton@csir.co.za; henkbert@tiscali.co.za

Corresponding author. Margaret B Nolan margaret.nolan@up.ac.za

Highlights

- Recombinant zona pellucida vaccination in horse mares is immunologically effective.
- A non-Freund's adjuvant is effective for zona pellucida immunocontraception.
- Side effects are minimal and transient.
- Anti-zona pellucida antibody titres are associated with ovarian activity in mares.

Abstract

Commercial and regulatory limitations associated with native porcine zona pellucida (pZP) vaccines formulated with Freund's adjuvants may be overcome by developing effective recombinant ZP vaccines (reZP) and identifying alternative adjuvant formulations. A two-part preparatory study used 15 geldings and identified potentially effective alternative adjuvant formulations based on anti-pZP antibody response following treatment with pZP formulated with Addavax (AddaVax ™, Invivogen), Quil A (Quil-A® Adjuvant, Invivogen), Quil A and Poly (I:C) (HMW VacciGrade™, Invivogen), Pet Gel A (Montanide™ Pet Gel A, Seppic) and Pet Gel A and Poly (I:C). Injection site reactions, rectal temperature and respiratory and heart rates were also monitored for three days post-treatment. Suitable anti-pZP antibody titres were seen in response to Pet Gel A and Pet Gel A and Poly (I:C). Subsequently in 31 mares, following administration of pZP, reZP and a combination of pZP and reZP proteins prepared in Pet Gel A and Poly (I:C), both serum anti-pZP and -reZP antibody responses were monitored. In addition, safety was assessed for up to seven days post-treatment by inspection and palpation of gluteal intramuscular injection sites and measurement of rectal temperature. The measured antibody titres in all treatment groups differed significantly to an adjuvant control group ($P<0.001$). Temporal changes in both anti-pZP and -reZP antibody titres in all ZP treatment groups were similar to patterns reported previously in various species vaccinated with pZP formulated with Freund's adjuvants. There were no differences in anti-pZP antibody titres between the pZP and reZP treated groups ($P>0.05$). Side effects were mild and transient in nature. This represents the first application of a reZP vaccine formulated with non-Freund's adjuvants evoking a similar antibody titre response to native pZP vaccination in mares.

Keywords antibody titres, zona pellucida proteins, immunocontraception, vaccine formulation, horse

1 Introduction

The induction of antibodies against zona pellucida (ZP) proteins for the control of fertility was first reported in 1972 [1]. In the absence of suitable adjuvant formulations ZP proteins are weak antigens [2]. Efficacy has, however, been demonstrated via the combination of native porcine zona pellucida (pZP) proteins formulated with Freund's modified complete adjuvant (FMCA) and Freund's incomplete adjuvant (FIA) for primary and booster immunisations, respectively. These immunocontraceptive vaccines have been used for more than 30 years in populations of both horses [3, 4] and white tailed deer [5] and for 18 years in African elephants [6, 7, 8]. In total, more than 90 zoo and wildlife species have been treated with pZP formulated with Freund's adjuvants to achieve fertility control [9]. In most, the primary treatment was followed by a booster after two or three weeks or, in African elephants, after five weeks [8]. The duration of the contraceptive effect was approximately one year in most species including African elephants and horses and single annual boosters were required to maintain this effect [10, 11, 12]. A liposomal pZP formulation containing cholesterol, a phospholipid and FMCA provided both a prolonged contraceptive effect in horses [13] and anti-pZP antibody titres in elephants [14]. Reversibility of this formulation has, however, not been demonstrated which may be problematic for the conservation of threatened species including the free-ranging African elephant.

The presumed immunocontraceptive mechanism of pZP vaccination involves antibody binding to the ZP sperm receptor sites and subsequent prevention of sperm-oocyte binding and fertilisation. Based on this supposition, pZP immunisation should not affect the hypothalamic-pituitary-gonadal axis, with continuation of cyclical ovarian activity [15]. Ovarian suppression in the equid has however, been reported in recent years [16]. It has been suggested variously that this suppression may be associated either with vaccine contamination by non-ZP proteins in the native derived pZP formulations or as a sequel to the use of Freund's adjuvant, although this has yet to be fully defined [17]. T-cell involvement has also been proposed as a cause of ovarian dysfunction subsequent to pZP vaccination

[18]. A clear link between ovarian functionality and a helper T-cell-mediated ($CD4^+$) response has been demonstrated in mice after ZP-vaccination, manifesting as either interstitial oophoritis excluding developing follicles and ovarian function interference [19] or as an effect on the developing follicles resulting in interference with ovarian function [20] dependent on the absence or presence of a detectable anti-ZP antibody response, respectively. A later study by Lloyd et al. (21) further demonstrated the association between a normal adaptive immune response (including a cell-mediated antibody response) and decreased fertility following ZP-vaccination. These studies collectively demonstrated the role of $CD4^+$ and antibodies in ZP-vaccine associated ovarian dysfunction (18). T-helper cells play a role in the production of antibodies by B-lymphocytes but it is the cytotoxic T-lymphocytes ($CD8^+$) that produce a direct cytotoxic effect. No overt confirmation of $CD8^+$ involvement in ovarian dysfunction subsequent to ZP- vaccination was demonstrated until Jooné et al in 2019 (22). Significant proliferation of $CD8^+$ T cells were detected in mares immunised with a pZP and reZP vaccine. Additionally, $CD8^+$ T cell levels were highly correlated with clinical measurements of ovarian cyclical activity in pZP vaccinated mares (22). An apparent link exists between the immunogenicity and contraceptive efficacy of a ZP vaccine and its effect on ovarian dysfunction (18).

Appropriate delivery systems for antigen presentation [23, 24] and effective cellular and humoral immune potentiators [23, 25] are required for ZP immunocontraception formulations. A recent approach to optimize vaccine immune responses utilises different adjuvant combinations that stimulate both Th1 and Th2 mediated responses [23, 26], which may be a useful prerequisite for successful ZP-based vaccination.

Previous investigations in mice and dogs have utilised alternative (i.e. non-Freund's) adjuvants including Pet Gel A, Alum and CP20, 961 in combination with ZP-antigens [27, 28]. In a murine model study [23] that used Pet Gel A for adjuvanting purposes, purified ZP3, the putative primary sperm receptor [29], was expressed with promiscuous T-cell epitopes of tetanus toxoid (TT-KK-ZP3) and ZP4 with a promiscuous T-cell epitope of bovine RNase

(bRNase-KK-ZP4). These two treatment formulations elicited respective high antibody titres as well as T-cell responses. A decrease in fertility was also reported. An additional treatment group was primed with pZP and received a booster with combined TT-KK-ZP3 and bRNase-KK-ZP4. This treatment protocol demonstrated the highest antibody titres for all antigens (pZP, ZP3 and ZP4). The application of combined TT-KK-ZP3 and bRNase-KK-ZP4 formulated with Freund's adjuvants in pony mares [16] resulted in an ineffective contraceptive effect coupled with a poor anti-pZP antibody titre response. Interestingly, the reZP formulation used in this study resulted in higher T-lymphocyte responses (both pZP-specific $CD4^+$ and $CD8^+$) than was seen in the pZP treated mares [22]. However no correlations existed between T-cell response and recorded clinical measures of cyclical ovarian activity of reZP treated mares (22). This same reZP formulation when administered in donkeys was associated with 100% contraceptive efficacy [30]. The post-synthesis treatment of the recombinant proteins used in the donkeys [30] differed from that used in the mare study [16, 22].

Injection site reactions associated with vaccine formulations containing Freund's adjuvants are well established in laboratory animals [31]. In the horse, probably the most-frequently studied species, as far as pZP-based immunocontraception is concerned, until very recently, injection site reactions following the use of Freund's adjuvants were rarely reported. A recent study in pony mares investigating pZP and reZP [16] formulated with FCMA (primary) and FIA (booster), reported injection site swelling and/or palpable changes in muscular density in over 95% of both treated and adjuvant control mares. Several developed overt sterile abscesses and this was observed more frequently in the reZP treated mares. The authors speculated that the higher frequency of abscesses may have been due to the presence of promiscuous T-cell epitopes in this formulation. A similar study in donkey jennies, which also compared pZP and reZP formulated with Freund's adjuvants, produced similar injection site reactions in both treated and adjuvant control groups. Similarly, more severe reactions were observed in the reZP-treated group [30]. Bechert et al. also reported localised reactions

varying in intensity and duration, including overt abscessation in mares treated with a pZP liposomal mixture formulated with FCMA in an aqueous solution [13]. This comparison of injection site reactions in response to differing formulations and treatment protocols was however, confounded by the differing methods of measurement used. Additionally, previous studies in wild horses [3, 4] seldom monitored injection site reactions on a daily basis and consequently, injection site reactions may have been under reported, particularly in the 2-3 weeks after inoculation.

Whilst Freunds' adjuvants with ZP-based vaccines are associated with high antibody titres and subsequent contraceptive efficacy [3], the identification of an appropriate alternative commercially-available adjuvant with a satisfactory safety profile is indicated. Whilst many of the conventional immunological adjuvants such as Freund's, bacterial toxins and non-purified crude agents (e.g. lipid A) typically induce strong immunogenic effects their administration also frequently induces adverse side-effects. Consequently any vaccine incorporating these adjuvants is unlikely to be approved by regulatory authorities [31, 32, 33, 34]. Vaccine adjuvants based on polymer technologies, such as Montanide™ Pet Gel A, are currently available for veterinary application and have been tested for safety and efficacy in several antigenic and animal models [35], including the horse [36]. Such adjuvants should be further investigated for use with immunocontraceptive vaccines.

Coupled with these issues, reliance on the native-derived proteins for pZP vaccine formulations remains an obstacle to efficient production (economical and immunogen purity), and its distribution and movement internationally [37, 38]. Several shortcomings associated with pZP vaccines, including extra-ZP ovarian proteins or other contaminants, stem from the use of the full complement of ZP proteins derived from a native source and it has been suggested that the continued successful application of ZP-based immunocontraception and standardisation of dose is contingent on the development of an effective recombinant formulation [16, 37, 38].

Recombinant ZP proteins have several compelling properties including improvements in antigen purity and structure [39]. The expression of recombinant proteins for vaccine antigen production using an *Escherichia coli* (*E. coli)* platform is well documented and reported benefits include high speed and yield of production, moderate production costs and scale-up capacity, no glycosylation and limited contamination risks in the form of endotoxins which can be addressed in purification processes [40].

The aims of this study were to identify a suitable non-Freund's adjuvant formulation for delivery of ZP proteins and to apply this formulation in a subsequent study to monitor antibody titres, injection site reactions and rectal temperature in mares following their immunisation with native pZP proteins, reZP proteins or a combination of pZP and reZP proteins.

2 Materials and Methods

2.1 Study 1

2.1.1 Subject selection, environment and management

Fifteen male horses (geldings) of mixed-breed type were studied from February to May 2016. Inclusion criteria were clinical health, adult status and normal body weight (range 306-458.5 kg). Horses were maintained at a single site at the South African Police Services Mounted Academy in Potchefstroom, North West Province, South Africa. Horses were maintained outdoors in four large paddocks providing ample space for freedom of movement and exhibition of normal behaviours.

2.1.2 Study design

Recruited horses were assigned to one of five treatment groups in this randomised controlled study. Treatments and measurements were initiated in February (d=0), repeated in April (d=35) and final measurements were taken in May (d=70).

2.1.3 Vaccine formulations

The antigen used in each formulation was native pZP (Trumpeter Farms and Veterinary Service, Winters, CA, USA) [3] and the dose per treatment was 100 µg.

Addavax (n=3): per dose (primary and booster) 500 µL squalene-based oil-in-water nano emulsion adjuvant (AddaVax ™, Invivogen, USA) was mixed with 500 µL phosphate buffered saline (PBS) containing the antigen.

Quil A (n=3): per dose 500 µg lyophilised purified saponin (Quil-A® Adjuvant, Invivogen, USA) reconstituted in 250 µL sterile water mixed with 500 µL PBS containing antigen and 250 µL physiological saline.

Quil A & Poly (I:C) (n=3): per dose 500 µg purified saponin reconstituted in 250 µL sterile water was mixed with 250 µL PBS containing antigen and 500 µg Polyinosinic-polycytidylic acid – TLR-3-based adjuvant (Poly (I:C) HMW VacciGrade™, Invivogen, USA) in 500 µL sterile water.

Pet Gel A (n=3): per dose 100 µL high molecular weight polyacrylic polymer in water adjuvant (10%; Montanide™ Pet Gel A, Seppic, France) was mixed with 500 µL PBS containing antigen and 400 µL physiological saline.

Pet Gel A & Poly (I:C) (n=3): per dose 100 µL Pet Gel A (10%) mixed with 250 µL PBS containing antigen, 500 µg Poly (I:C) in 500 µL sterile water and 150 µL physiological saline.

2.1.4 Vaccine administration

Formulations were prepared on site and volumes were standardised at 1 mL per treatment. Primary vaccinations were administered in February (d=0) and single boosters 35 days later (d=35). All vaccines were administered by deep intramuscular injection (19-gauge needle) into the gluteal muscle mass. Boosters were administered into the contralateral musculature.

2.1.5 Sample collection and observations.

Blood samples were collected by jugular venipuncture at d=0, d=35 and d=70 for measurement of serum anti-pZP antibody titres. Samples were centrifuged and serum stored at -20° C until assayed. Prior to and for three days following treatment, safety and side effects were assessed. The injection sites were assessed subjectively by visual inspection and palpation for changes including heat and swelling and scored using a three point scale (category 0 = no reaction; 1 = palpable reaction; 2 = visible reaction with or without pain upon palpation). Rectal temperatures were measured using a digital thermometer (Kruuse, Langeskov, Denmark) and respiratory and heart rates were recorded.

2.2 Study 2

2.2.1 Subject selection, environment and management

Thirty one mixed-breed horse mares (light body type: Arabian, Quarter Horse, Draught and Thoroughbred cross; age: 2-10 y) were studied from November 2016 to May 2017, during the physiological breeding season in the southern hemisphere. Inclusion criteria were oestrous cyclicity, non-pregnant status, good clinical and reproductive health and no previous immunocontraceptive exposure. Mares were maintained on a single extensive mountainous grassland site (3000 ha) in pre-existing groups. The study site was located near Underberg, KwaZulu-Natal Province, South Africa.

2.2.2 Study design

Recruited subjects were stratified by body condition scores (BCS 1-9) [41], parity and age and assigned to one of four treatment groups in this randomised controlled study. Treatments and measurements were initiated in December (d=0) and repeated in January (d=35) and February (d=70) and further measurements were taken in March (d=105) and May (d=175).

2.2.3 Antigens used

Native pZP vaccine (Trumpeter Farms and Veterinary Service, Winters, CA, USA) was prepared according to standard methods [3]. Recombinant ZP3 and ZP4 proteins (reZP; supplied by Biomanufacturing Technologies, Biosciences, CSIR, South Africa) were expressed in *E. coli* according to Gupta et al. [24] with several modifications. Briefly, the antigen sequences were produced according to GenBank accession numbers NP_999058 and NP_999210, respectively, encoding porcine ZP3 (amino acid (aa) residues 20–421) and porcine ZP4 (aa residues 23–463) without the signal peptide and transmembrane-like domain. Zona pellucida 3 contained the tetanus toxin (TT; aa residues 830–844) at its N-terminus. Similarly, ZP4 incorporated the promiscuous T-cell epitope of bovine RNase (bRNase; aa residues 94–104). Both expressed products were confirmed by LC-MS peptide mapping. Doses of antigen used per immunisation were 100 µg and 500 µg (250 µg ZP3 and 250 µg ZP4) for pZP and reZP, respectively.

2.2.4 Vaccine formulations

The antigens were formulated in 6% Pet Gel A and 500 µg Poly (I:C) and were lyophilised in multi-vials. The same formulation was used for the adjuvant control group without addition of antigen.

2.2.5 Vaccine administration

Vaccines were reconstituted with sterile injection water immediately prior to administration to provide a treatment volume of 1 mL. All vaccines were administered by deep intramuscular injection (19-gauge needle) into the gluteal muscle mass. Boosters were administered into the contralateral musculature.

Adjuvant control group mares (n=8) were treated with adjuvant on d=35 with an identical booster on d=70.

The pZP only group (n=7) were treated on d=35 with 100µg of native pZP and adjuvant with an identical booster on d=70.

The reZP only group (n=8) were treated on d=0 with 500µg reZP and adjuvant and again on d=35 and d=70 with identical boosters. The reZP only treatment was started earlier so all groups' period of assumed maximal antibody titre (d=105) would align.

The pZP & reZP group (n=8) were treated on d=35 with 100 µg pZP and adjuvant and on d=70 with 500µg reZP and adjuvant.

2.2.6 Sample collection and observations

Blood samples were collected by jugular venipuncture at d=0, d=35 d=70, d=105 and d=175 for measurement of serum anti-ZP antibody titres. Samples were centrifuged and serum stored at -20° C until assayed. Prior to and for up to seven days following treatment, safety and side effects (injection site reactions and rectal temperature) were assessed as described in Study 1. Additionally, a concurrent investigation [42] in Study 2 monitored ovarian function subsequent to immunocontraception. Briefly, transrectal palpation and ultrasonography of the reproductive tract was performed at d=0, d=35, d=70, d=105 and d=175. Ovarian activity or inactivity was described dependent on detection of the presence or absence of follicles >15 mm, ovarian volume > or <25 cm^3 (prolate ellipsoid formula) and serum progesterone levels > or <1 ng/mL.

2.3 Anti-pZP and reZP antibody titre assays (Study 1 and 2)

Anti-ZP antibody response was measured by enzyme immunoassay (EIA), using a modification of a method previously described [16]. All tested sera were assayed in duplicate and expressed as a proportion of a positive reference standard at the same dilution rate. For the anti-pZP antibody assay (Study 2) the positive reference standard consisted of pooled sera from the pZP only treatment group at time of assumed maximal titre (d=105). For the anti-reZP antibody assay (Study 2) and anti-pZP antibody assay (Study 1) the positive reference standard consisted of previously stored pooled sera from mares treated with a pZP vaccine containing Freund's adjuvants [16]. Ninety six well plates (Nunc Immunoplate F76 Maxisorp, South Africa) were incubated at 2-8 °C for 16 h with 1 µg (pZP or reZP (0.5 µg ZP3 and 0.5 µg ZP4)) in 100 µL coating buffer (2.94% $NaHCO_3$, 1.59% Na_2CO_3, pH 9.6) per well. Plates were washed with PBS containing 0.05% Tween 20 and then blocked with 0.03% BSA in PBS for 16 h at 2-8 °C. Plates were then incubated with serial dilutions of standard and test serum samples at 37 °C for 1 h (anti-pZP antibody assay (Study 1) 1:1000 to 1:16000 for test samples and 1:1000 to 1:64,000 for positive reference serum; anti-pZP antibody assay (Study 2) 1:250 to 1:4000 for test samples and 1:250 to 1:16,000 for positive reference serum; anti-reZP antibody assay 1:8000 to 1:128000 for test samples and 1:4000 to 1:512,000 for positive reference serum). Wells containing PBS were used as blanks (negative controls). After washing, antibodies were detected by incubating plates with recombinant protein G-horseradish peroxidase (LTC Tech South Africa, Johannesburg, South Africa) at 37 °C for 1 h. After further washing, plates were developed with trimethylene blue (SureBlue™). The reaction was stopped by adding 50 µL of 2 mol/L H_2SO_4 per well. Absorbance at 450 nm was measured using a microplate photometer (Multiskan™ FC). Antibody response was measured as the mean sample absorbance (minus blank) expressed as a proportion of the mean absorbance (minus blank) of the positive reference standard at the same dilution for each plate. The overall proportion positive was calculated as the average value over three dilutions. Intra- and inter-assay coefficients of variation were 9.07%

and 16.32% for the anti-pZP antibody (Study 1), 4.02% and 10.83% for the anti-pZP antibody (Study 2) and 5.72% and 7.79% for the anti-reZP antibody assays, respectively.

2.4 Data analyses (Study 1 and 2)

Data were assessed for normality through the plotting of histograms, calculation of descriptive statistics and the Shapiro-Wilk test for normality.

Quantitative data were analysed using mixed effect linear regression. For statistical interrogation of group differences of categorical safety data, injection site reactions were reclassified as either present or absent. Similarly, elevated rectal temperatures were reclassified as ≥39 °C or <39 °C and were compared among treatment groups using mixed effects logistic regression (Study 2). Regression models included fixed effect terms for treatment group, sampling time (categorical with three/five levels) and a group by time interaction. Horse age and BCS were also included as fixed effects. Horse was included as a random effect and a first-order autoregressive correlation structure was used to account for repeated sampling. *Post-hoc* tests in the mixed-effects models were adjusted using the least significant differences (LSD) method. Additionally, binomial logistic regression analysis was performed to investigate anti-pZP and –reZP antibody titres as a predictor of ovarian inactivity in response to treatment. A first-order autoregressive correlation structure was used to account for repeated sampling. . Statistical testing was performed using commercially available software (IBM SPSS Statistics Version 25) and significance was set at $P \leq 0.05$.

3 Results

3.1 Study 1

3.1.1 Anti-pZP antibody titre

Treatment, time and the treatment by time interaction all had a significant effect on anti-pZP antibody titres collected over the entire study (All P<0.001) (Figure 1). Overall the anti-pZP antibody titres of the Addavax group were significantly lower than the Quil A (P=0.028), Quil A & Poly (I:C) (P=0.011), Pet Gel A (P<0.001) and Pet Gel A & Poly (I:C) (P<0.001) treated groups. The Quil A and Quil A & Poly (I:C) treated groups were significantly lower than the Pet Gel A (P=0.008 and P=0.020, respectively) and Pet Gel A & Poly (I:C) groups (P=0.007 and P=0.017, respectively). No differences were evident between the Quil A and Quil A & Poly (I:C) treated horses (P=0.591) and the Pet Gel A and Pet Gel A & Poly (I:C) treated groups (P=0.933).

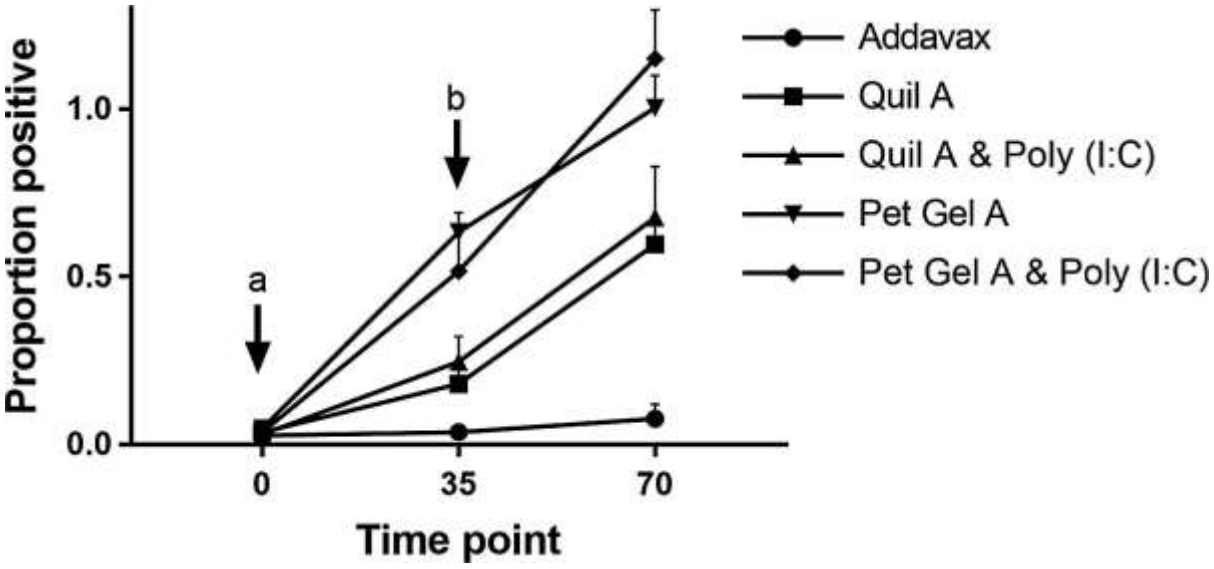

Fig. 1. Study 1 (geldings) mean anti-pZP antibody response expressed as a proportion of the positive standard (+s.e.) for all treatment groups (Addavax: n = 3; Quil A: n = 3; Quil A & Poly (I:C): n = 3; Pet Gel A: n = 3; Pet Gel A & Poly (I:C): n = 3) at successive time points 0 (d = 0), 35 (d = 35) and 70 (d = 70). a: primary vaccination; b: booster vaccination.

3.1.2 Injection site reactions, rectal temperature, respiratory rate and heart rate

Following the primary vaccination there were no notable increases in rectal temperature. Following the booster, however, increases in temperature were observed in the Quil A & Poly (I:C) and both Pet Gel A groups. The highest rectal temperatures were measured in the Pet Gel A & Poly (I:C) treatment group. By the second day post-treatment all temperatures had returned to normal levels with the exception of the Pet Gel A & Poly (I:C) treated group which returned to normal levels three days post-treatment. An increase in localised swelling was seen in the Quil A group and the two Pet Gel A groups following the primary treatment. The booster was associated with noticeable swellings in all groups except the Addavax group. The two Pet Gel A groups displayed more injection site reactions, these, however, were no longer detectable within a week of treatment administration.

Respiratory rate increases were not evident following administration of any formulation but rather seemed to increase in association with increased environmental temperatures (environmental temperature reached a 39 °C maximum during the primary treatment administration on February 29th 2016 and subsequently a 26 °C maximum on the first day of the booster treatment administration on April 4th 2016). Heart rates remained consistent in all groups during both observation periods

3.2 Study 2

3.2.1 Anti-pZP antibody titre

Treatment, time and the treatment by time interaction all had a significant effect on anti-pZP antibody titres (all $P<0.001$). The fixed effect terms of age and BCS had no effect on anti-pZP antibody titres ($P=0.474$, $P=0.085$, respectively). Overall, anti-pZP antibody titres changed significantly at each time point from d=0 until d=75 ($P<0.001$), steadily increasing until d=105 followed by a decline at d=175 (Figure 2). No significant differences were measured between pZP only and reZP only mares but reZP only and pZP only treated

mares' titres differed to pZP & reZP treated mares, with lower concentrations in pZP & reZP treated mares (P=0.009, P<0.001, respectively) (Figure 3).

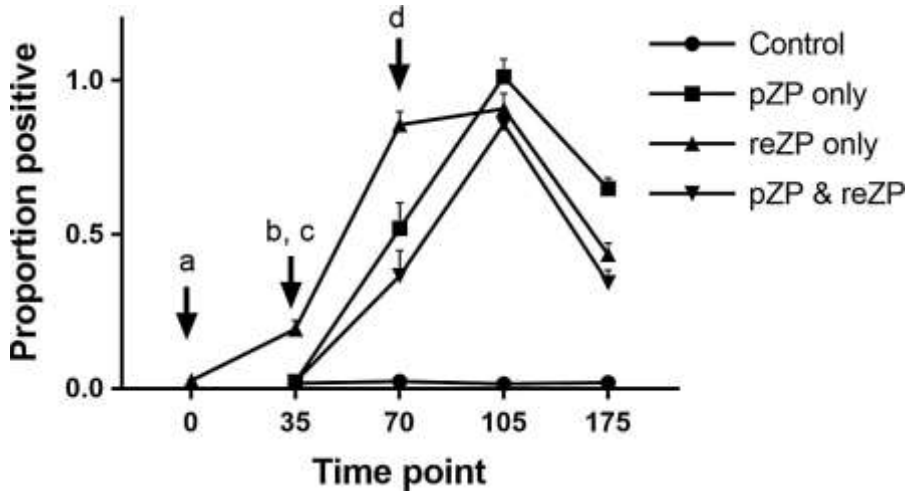

Fig. 2. Study 2 (mares) mean anti-pZP antibody response expressed as a proportion of the positive standard (+s.e.) for all treatment groups (Adjuvant control: n = 8; pZP only: n = 7; reZP only: n = 8; pZP & reZP: n = 8) at successive time points 0 (d = 0: reZP only), 35 (d = 35: reZP; d = 0 values plotted for all other groups), 70 (d = 70), 105 (d = 105) and 175 (d = 175). a: primary vaccination (reZP only); b: booster vaccination (reZP only); c: primary vaccination (adjuvant control, pZP only, pZP & reZP); d: booster vaccination (all groups).

Pairwise Comparisons[a]

(I) GROUP_PZP	(J) GROUP_PZP	Mean Difference (I-J)	Std. Error	df	Sig.[e]	95% Confidence Interval for Difference[e]	
						Lower Bound	Upper Bound
Group 1	Group 2	-.538[*,c,d]	.036	27.166	.000	-.613	-.463
	Group 3	-.467[*,c]	.033	22.440	.000	-.536	-.399
	Group 4	-.374[*,c,d]	.034	25.194	.000	-.443	-.304
Group 2	Group 1	.538[*,c,d]	.036	27.166	.000	.463	.613
	Group 3	.071[c]	.035	24.492	.055	-.002	.143
	Group 4	.165[*,c,d]	.036	26.930	.000	.090	.239
Group 3	Group 1	.467[*,d]	.033	22.440	.000	.399	.536
	Group 2	-.071[d]	.035	24.492	.055	-.143	.002
	Group 4	.094[*,d]	.033	22.067	.009	.026	.162
Group 4	Group 1	.374[*,c,d]	.034	25.194	.000	.304	.443
	Group 2	-.165[*,c,d]	.036	26.930	.000	-.239	-.090
	Group 3	-.094[*,c]	.033	22.067	.009	-.162	-.026

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Dependent Variable: pZP_Titres.

c. An estimate of the modified population marginal mean (I).

d. An estimate of the modified population marginal mean (J).

e. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Fig. 3. Study 2 (mares) anti-pZP antibody response group comparisons for all treatment groups (Group 1 Adjuvant control: n = 8; Group 2 pZP only: n = 7; Group 3 reZP only: n = 8; Group 4 pZP & reZP: n = 8) based on regression analysis including fixed effect terms for treatment group, sampling time (categorical with five levels), a group by time interaction, age and BCS. Horse was included as a random effect and a first-order autoregressive correlation structure was used to account for repeated sampling. *Post-hoc* tests in the mixed-effects models were adjusted using the least significant differences (LSD) method.

3.2.2 Anti-reZP antibody titre

Treatment, time and the treatment by time interaction all had a significant effect on anti-reZP antibody titres (all P<0.001). The fixed effect terms of age and BCS had no effect on anti-reZP antibody titres (P=0.156, P=0.478, respectively). Overall, anti-reZP antibody titres changed significantly at each time point from d=0 until d=175 (P<0.001), following a similar temporal pattern to that for anti-pZP antibody titres (Figure 4). In this instance the reZP only treated mares showed significantly higher titres than both pZP only and pZP & reZP treated mares (both P<0.001). The pZP only group and pZP & reZP group also differed (P=0.037) (Figure 5).

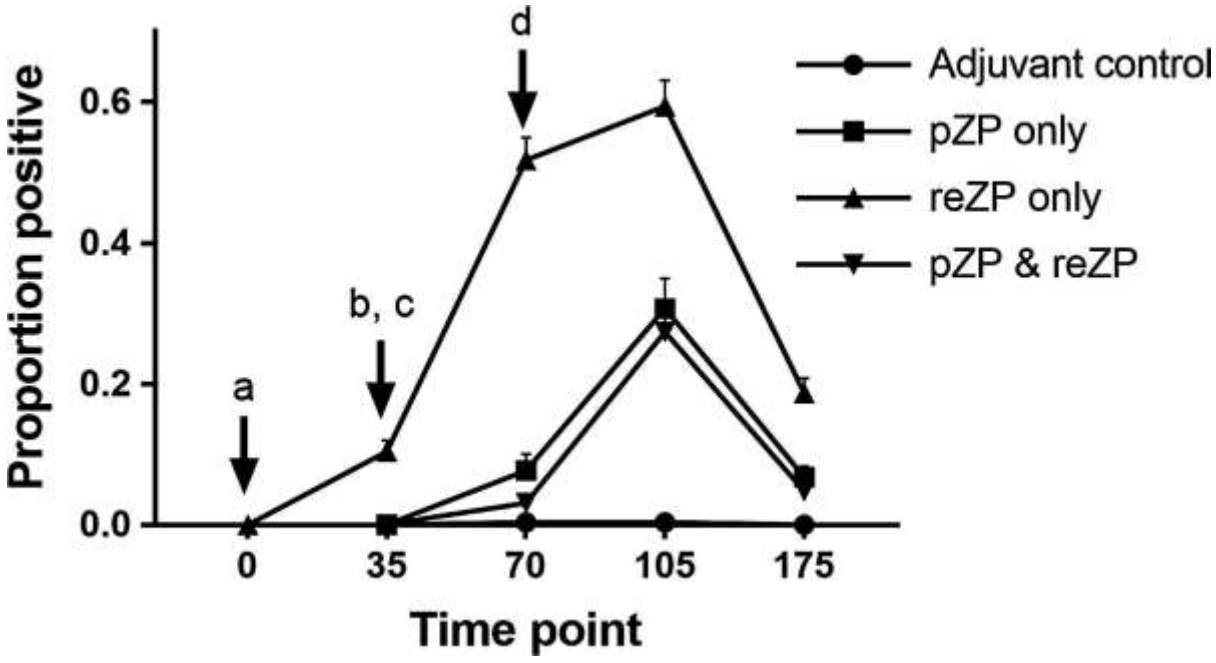

Fig. 4. Study 2 (mares) mean anti-reZP antibody response expressed as a proportion of the positive standard (+s.e.) for all treatment groups (Adjuvant control: n = 8; pZP only: n = 7; reZP only: n = 8; pZP & reZP only: n = 8) at successive time points 0 (d = 0: reZP only), 35 (d = 35:reZP; d = 0 values plotted for all other groups), 70 (d = 70), 105 (d = 105) and 175 (d = 175). a: primary vaccination (reZP only); b: booster vaccination (reZP only); c: primary vaccination (adjuvant control, pZP only, pZP & reZP); d: booster vaccination (all groups).

Pairwise Comparisons[a]

(I) GROUP_PZP	(J) GROUP_PZP	Mean Difference (I-J)	Std. Error	df	Sig.[e]	95% Confidence Interval for Difference[e]	
						Lower Bound	Upper Bound
Group 1	Group 2	-.127*,c,d	.017	55.170	.000	-.161	-.093
	Group 3	-.285*,c	.015	44.658	.000	-.316	-.254
	Group 4	-.090*,c,d	.016	51.511	.000	-.122	-.058
Group 2	Group 1	.127*,c,d	.017	55.170	.000	.093	.161
	Group 3	-.158*,c	.017	48.448	.000	-.191	-.125
	Group 4	.037*,c,d	.017	54.515	.037	.002	.071
Group 3	Group 1	.285*,d	.015	44.658	.000	.254	.316
	Group 2	.158*,d	.017	48.448	.000	.125	.191
	Group 4	.195*,d	.015	43.929	.000	.164	.226
Group 4	Group 1	.090*,c,d	.016	51.511	.000	.058	.122
	Group 2	-.037*,c,d	.017	54.515	.037	-.071	-.002
	Group 3	-.195*,c	.015	43.929	.000	-.226	-.164

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Dependent Variable: REZP_ZP.

c. An estimate of the modified population marginal mean (I).

d. An estimate of the modified population marginal mean (J).

e. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Fig. 5. Study 2 (mares) anti-reZP antibody response group comparisons for all treatment groups (Group 1 Adjuvant control: n = 8; Group 2 pZP only: n = 7; Group 3 reZP only: n = 8; Group 4 pZP & reZP: n = 8) based on regression analysis including fixed effect terms for treatment group, sampling time (categorical with five levels), a group by time interaction, age and BCS. Horse was included as a random effect and a first-order autoregressive correlation structure was used to account for repeated sampling. *Post-hoc* tests in the mixed-effects models were adjusted using the least significant differences (LSD) method.

3.2.3 Injection site reactions and rectal temperature

Injection site reactions were observed in 22%, 55%, 46% and 47% of examinations in adjuvant control, pZP only, reZP only and pZP & reZP treatment groups, respectively. Elevated rectal temperatures (≥38.4 °C) occurred in 25%, 25%, 33% and 29% of examinations in adjuvant control, pZP only, reZP only and pZP & reZP treatment groups, respectively.

Treatment, time and the treatment by time interaction all had a significant effect on the incidence of injection site reactions (all $P<0.001$). Considerably more injection site reactions occurred in the reZP only mares compared to both the adjuvant control and pZP only mares (both $P<0.05$). No other significant treatment group differences were seen. The occurrence

of injection site reactions increased with each subsequent treatment administration (P<0.05). All reactions were both mild (category 1=97.5%) and transient, resolving within seven days of treatment administration. A similar pattern was observed for the post-treatment occurrence of elevated rectal temperatures. Treatment, time and the treatment by time interaction all had significant effects (all P<0.001), with a higher incidence of elevated temperature with each subsequent treatment administration (P<0.05), however, all had returned to within normal limits within seven days. No significant differences were seen between individual treatment groups.

3.2.4 Ovarian activity

An assessment of the causal relationship indicated that anti-pZP and –reZP antibody titres were a significant predictor of ovarian inactivity (P=0.001 95% CI [0.644, 2.537], P=0.001, 95% CI [1.321, 4.758], respectively).

4 Discussion

The adjuvant combinations chosen for investigation were selected for their respective immunomodulatory components according to manufacturer's specification; Pet Gel A as an antigen carrier and cell and non-cell mediated potentiator [36, 43]; Addavax [44] and Quil A [45] for Th1 and Th2 response and Poly (I:C) as a TLR-3 agonist [46].

Study 1 showed that both Pet Gel A groups performed better than the other groups in invoking anti-pZP antibody titres. Furthermore, in combination with Poly (I:C), this increased the antibody response to native pZP. There was no significant difference in titres achieved between the two Pet Gel A groups, but T-cell proliferation analysis notwithstanding, further investigations of the combined Pet Gel A and Poly (I:C) were indicated.

Subsequent discussions with the manufacturers (Seppic, France) suggested that Pet Gel A concentration could be reduced from 10% to a 6% polymeric preparation without affecting

overall antibody response. The side effects observed with both Pet Gel A formulations were confined to rapidly resolving local swelling and temperature reactions. These two variables, unlike heart and respiratory rate measurements, proved most informative in monitoring post-treatment reactions.

The results of this preparatory study informed the design and formulations used subsequently in Study 2.

Study 2 is the first to describe the immune response of horses following vaccination with pZP and reZP proteins formulated with non-Freund's adjuvants. In this study, anti-pZP antibody titres following vaccination with native pZP, reZP or pZP & reZP formulated with a combination adjuvant of Pet Gel A and Poly (I:C) showed temporal changes similar to previous reports in mares vaccinated with pZP formulated with Freund's adjuvants [16]. Furthermore, there was no difference in anti-pZP titres in mares treated with pZP only or reZP only formulations. The different treatment protocols between groups used in this study may arguably have played a role in the high anti-pZP antibody titre response in the reZP only group. Multiple boosters are reportedly required for non-glycosolated synthetic vaccines to improve immunogenicity (47).Previously, this research group reported a poor anti-pZP antibody response in pony mares following reZP treatment [16]. In the current study, higher anti-reZP antibody titres were seen in the reZP only treated mares than in those receiving the other ZP treatments. The reZP vaccine used in the earlier study was sourced from a different laboratory and manufactured differently, formulated with Freund's adjuvant and only a single booster treatment was administered. It has been previously asserted that 70-80% of the pZP antigen is likely accounted for by ZP3, and when injected, the mare may produce substantially more antibodies against ZP3 than the other ZP proteins. The pZP only mares in the current study supported this assertion by producing high anti-reZP antibody titres [48, 49]. The higher anti-reZP titres in the reZP only group may be associated with the presence of TT and BRNase epitopes. The additional booster in this instance did not appear to bolster the anti-reZP antibody titre response. After a single booster treatment, the anti-reZP

antibody titres at d=70 measured in the reZP treated mares was already significantly higher than all other groups at a corresponding measurement (d=105). The reZP only mares maintained higher anti-reZP titres until the end of the observation period, which may be a feature of the additional booster. Mares primed with pZP and boosted with reZP did not achieve the highest anti-reZP or anti-pZP antibody titres in contradiction to a previous report where mice were used as a model species [27].

Reports of undesirable side effects vary with the use of Freund's adjuvants for ZP based immunocontraception. This may be at least partially due to the limitations associated with clinical monitoring in feral horse populations rather than their absence. The current study, similar to more recent investigations [16, 30], monitored injection sites closely, however, in this instance there was minimal local reactivity and all reactions and elevated rectal temperatures were mild and transient in nature.

The commercial and regulatory limitations of both native pZP immunocontraceptive vaccines and Freund's adjuvants may be overcome through the use of reZP proteins expressed with promiscuous T-cell epitopes of tetanus toxoid and bovine RNase formulated with a commercially available polyacrylic polymer in water adjuvant that provides good antigen delivery (6 %; Montanide™ Pet Gel A) [42, 43] and a TLR-3 agonist (500 µg Poly (I:C)) [46].

An interesting, though not entirely unprecedented, finding [3] was the strongly significant relationship with anti-ZP antibody titres and ovarian inactivity. However, the contraceptive efficacy of the formulations used in this study requires further investigation. Additionally, the cell-mediated immune response following the use of these novel vaccine formulations should be assessed.

In conclusion, this was the first reported administration of a reZP vaccine formulated with non-Freund's adjuvant evoking a similar antibody titre response to a native pZP vaccine in mares.

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Ethical animal research

These studies complied with ARRIVE guidelines and were approved by the University of Pretoria Animal Ethics Committee (V051-13; V124-16)

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Declarations of interest:

None

Authorship

M.B. Nolan and M.L. Schulman contributed to the study design, data collection, data analysis and interpretation, preparation and final approval of the manuscript. H.J Bertschinger contributed to the study design, data analysis and interpretation, preparation

and final approval of the manuscript. A.E. Botha contributed to the data collection and final approval of the manuscript. A.M. Human contributed to sample analyses, pZP preparation and final approval of the manuscript. R. Roth and M. Crampton contributed to reZP preparation and final approval of the manuscript. All authors attest they meet the ICMJE criteria for authorship.

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