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

Author names and affiliations. 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

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

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. In a two-part study, a preparatory trial using 15 geldings 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) (500 µg Poly(I:C) HMW VacciGrade™, Invivogen), Pet Gel A (10%; Montanide™ Pet Gel A, Seppic) and Pet Gel A and Poly (I:C). Injection site reactions, body temperature and respiration and heart rates were also monitored. Sufficient 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 combined pZP and reZP proteins prepared in 6% Pet Gel A and 500 µg Poly (I:C), their serum anti-pZP and -

reZP antibody responses were monitored. In addition, safety was assessed for seven days post-treatment by inspection and palpation of gluteal intramuscular injection sites and measurement of body 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 pZP and reZP treated mares ($P>0.05$). Side effects were mild and transient in nature. This represents the first application of a reZP vaccine evoking a similar antibody titre response to native pZP vaccine in mares. Furthermore, incorporation of a novel non-Freund's adjuvant provided an alternative effective formulation for ZP-based immunocontraception.

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

Introduction

The induction of antibodies against zona pellucida (ZP) proteins for the inhibition 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 for more than 30 years in populations of 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 is approximately one year in most species including African elephants and horses and single annual boosters are administered to maintain this effect [10, 11, 12]. A liposomal pZP formulation containing cholesterol, a phospholipid and FMCA has 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, thereby permitting continuation of cyclical ovarian activity [15]. Ovarian suppression has however been reported in recent years [16, 17]. 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 an aspect of formulations with Freund's adjuvant, although this has yet to be fully defined [18].

Appropriate delivery systems for their antigen presentation properties [19, 20] and effective cellular and humoral immune potentiators [19, 21] are required for ZP immunocontraception formulations. A recent approach to optimize vaccine immune responses is the use of different adjuvant combinations that stimulate both Th1 and Th2 mediated responses [19, 22],which may be useful and necessary for 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 [23, 24]. In a murine model study [23] that used Pet Gel A for adjuvating purposes, purified ZP3, the putative primary sperm receptor [25], 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). The epitopes of tetanus toxoid and bovine RNase served as a carrier for the protein antigen and toll-like receptor (TLR) antagonist, respectively. These two treatment formulations elicited both high anti-ZP3 and -ZP4 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 in this study, this reZP formulation resulted in higher T-lymphocyte responses (both pZP-specific $CD4^+$and $CD8^+$) than was seen in the pZP treated mares [26]. This same formulation in donkeys was associated with ovarian suppression in 77.8% of treated animals and a contraceptive efficacy of 100% [17].Cytotoxic T-cell involvement has been proposed as a cause of ovarian dysfunction subsequent to pZP-based vaccination [27]. .

Commented [PMS1]: Which? reZP or pZP or both?

Commented [PMS2]: Does this not go above around lines 80-83 when you describe ovarian suppression? Here you are talking about the ABT and contraceptive response

Injection site reactions associated with vaccine formulations containing Freund's adjuvants are well established in laboratory animals [28]. In the horse, probably the most-frequently studied species, as far as pZP 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 and FIA, 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 formula. 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 [17]. Bechert et al. also reported localised reactions varying in intensity and duration, including overt abscessation in mares treated with a pZP liposome mixture formulated with FCMA in an aqueous solution [13].

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. Coupled with these issues, reliance on the native-derived proteins for pZP vaccine formulations remains an obstacle to both efficient production (economical and immunogen purity) and its distribution and movement internationally [29, 30].

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

Commented [PMS3]: ZP proteins?

Materials and Methods

Study 1

Subject selection, environment and management

Fifteen male horses (geldings) of mixed-breed type were studied from February to May 2016. Inclusion criteria were good physical 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.

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).

Vaccine formulations

The antigen used in each formulation was native pZP (Trumpeter Farms and Veterinary Service, Winters, California, USA) 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 polymeric 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.

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 into the gluteal muscle mass. Boosters were administered into the contralateral musculature.

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). Body temperatures were measured *per* rectum using a digital thermometer (Kruuse, Denmark) and respiration and heart rates were recorded.

Study 2

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 physical 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.

Study design

Recruited subjects were stratified by body condition scores (BCS 1-9) [31], 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).

Antigens used

Native pZP vaccine (Trumpeter Farms and Veterinary Service, Winters, California, USA) was prepared according to standard methods [2]. Recombinant ZP3 and ZP4 proteins containing epitopes of tetanus toxoid and bovine RNase (reZP; supplied by Biosciences, CSIR, South Africa) were expressed in *E. coli* according to Gupta et al. [23] with several modifications. Doses of antigen used per immunisation were 100 µg and 500 µg (250 µg ZP3 and 250 µg ZP4) for pZP and reZP, respectively.

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 adjuvant control group without addition of antigen.

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 into the gluteal muscle mass. Boosters were administered into the contralateral musculature.

Adjuvant control mares (n=8) were treated on d=35 with a booster on d=70.

The pZP only mares (n=7) were treated on d=35 with a booster on d=70.

The reZP only mares (n=8) were treated on d=0, d=35 and d=70.

The pZP & reZP mares (n=8) were treated on d=35 and d=70.

Sample collection and observations

Blood samples from all mares were collected by jugular venipuncture at d=0, d=35 d=70, d=105 and d=175 for measurement of serum anti-pZP antibody titres. Samples were centrifuged and serum stored at -20° C until assayed. Prior to and for seven days following treatment, safety and side effects (injection site reactions and body temperature) were assessed as described in Study 1.

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 (mare trial) 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 (mare trial) and anti-pZP antibody assay (gelding trail) the positive reference standard consisted of previously stored pooled sera from mares treated with a pZP vaccine containing Freund's adjuvants [15]. 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 (gelding) 1:1000 to 1:16000 for test samples and 1:1000 to 1:64,000 for positive reference serum; anti-pZP antibody assay (mare) 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

Commented [PMS4]: Study 2

Commented [PMS5]: Study 2

Commented [PMS6]: Stick to Study 1 and Study 2

Commented [PMS7]: Study 1

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 (gelding), 4.02% and 10.83% for the anti-pZP antibody (mare) and 5.72% and 7.79% for the anti-reZP antibody assays, respectively.

Commented [PMS8]: Study 1

Commented [PMS9]: Study 2

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 data, injection site reactions were reclassified as either present or absent. Similarly, elevated body 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 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. Anti-ZP antibody titres at each sampling time were compared among groups using one-way ANOVA with *post-hoc* multiple comparisons adjusted using Bonferroni correction of P values (Study 2). Pairwise correlations for anti-ZP antibody titres and all other measurements were

assessed using Spearman's rho or Pearson's correlation coefficient as applicable (Study 2). Statistical testing was performed using commercially available software (IBM SPSS Statistics Version 25) and significance was set at P≤0.05.

Results

Study 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).

Injection site reactions, body temperature, respiration rate and heart rate

Following the primary vaccination there were no notable increases in body 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 body 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 visible 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

Study 2

Anti-pZP antibody titre

Treatment, time and the treatment by time interaction all had a significant effect on anti-pZP antibody titres (all $P<0.001$). 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). All ZP treatment group titres assessed at the time of assumed maximal antibody titres (d=105) differed significantly to the control group (all $P<0.001$). No significant differences were measured between pZP only and reZP only mares but pZP only and pZP & reZP treated mares' titres differed, with lower concentrations in pZP & reZP treated mares ($P<0.001$).

Anti-reZP antibody titre

Treatment, time and the treatment by time interaction all had a significant effect on anti-reZP antibody titres (all $P<0.001$). 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 3). Again, all treatment group titres assessed at the time of assumed maximal antibody titres (d=105) differed significantly to the control group (all $P<0.001$). In this instance the reZP only treated mares showed significantly higher titres than

306 both pZP only and pZP & reZP treated mares (P<0.001). No such differences were seen
307 between pZP only and pZP & reZP treated mares.

308 ***Injection site reactions and body temperature***

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

313 Treatment, time and the treatment by time interaction all had a significant effect on the
314 incidence of injection site reactions (all P<0.001). Considerably more injection site reactions
315 occurred in the reZP only mares compared to both the adjuvant control and pZP only mares
316 (both P<0.05). No other significant treatment group differences were seen. Injection site
317 reactions occurred with increased frequency with each subsequent treatment administration
318 (P<0.05). All reactions were both mild (category 1=97.5%) and transient, resolving within
319 seven days of treatment administration. A similar pattern was observed for the post-
320 treatment occurrence of elevated temperatures. Treatment, time and the treatment by time
321 interaction all had significant effects (all P<0.001), with a higher incidence of elevated
322 temperature with each subsequent treatment administration (P<0.05), however all had
323 returned to within normal limits within seven days. No significant differences were seen
324 between individual treatment groups.

325 ***Pairwise correlations***

326 Anti-pZP and anti-reZP antibody responses were not significantly correlated to either mare
327 age (P =0.975, P=0.971) or BCS (P =0.641, P=0.768). A concurrent study [32] had
328 monitored ovarian function subsequent to immunocontraception *via* clinical observation and
329 measurement of serum progesterone and anti-Müllerian hormone (AMH). A significant
330 negative correlation was seen with anti-pZP antibody titres and both ovarian activity (ρ=-
331 0.381, P<0.001) and serum AMH concentrations (ρ=-0.271, P=0.002). Similarly, a negative

correlation was seen for anti-reZP antibody titres with both ovarian activity (ρ=-0.412, P<0.001) and AMH (ρ=-0.192, P<0.05).

Discussion

The adjuvant combinations chosen for investigation were selected for their antigen carrying function (Pet Gel A), TLR agonist action (Addavax: TLR-4 and TLR-7, Poly (I:C): TLR-3) and documented effective cell and non-cell mediated potentiation (Quil A, Pet Gel A).

Study 1 showed that both Pet Gel A groups performed better than the other groups in invoking anti-ZP 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 the 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 (measuring rectal temperature and subjective assessment of injection sites), 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 in the subsequent 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 any of the pZP only, reZP only or pZP & reZP formulations. 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 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 proteins. The pZP only mares in the current study supported this assertion by producing high anti-reZP antibody titres [33]. The higher anti-reZP titres in the reZP only group may also be a feature of the additional booster or associated with the presence of TT and BRNase epitopes.

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 monitored injection sites closely and showed minimal local reactivity and all reactions and elevated body 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 with a commercially available polyacrylic polymer in water adjuvant that provides good antigen delivery (Montanide™ Pet Gel A) [34, 35] and a TLR-3 agonist (Poly(I:C)) [36].

An interesting, though not unprecedented, finding [3] was the highly significant correlation of both anti-pZP and -reZP antibody titres with ovarian activity. 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 is the first application of a reZP vaccine evoking a similar antibody titre response to a native pZP vaccine in mares. Furthermore, incorporation of a novel non-Freund's adjuvant provided an alternative effective formulation for ZP-based immunocontraception.

Funding

This study was funded by the Technology Innovation Agency, Pretoria, South Africa. TAHC12-0042: Immunocontraception as a means of population control in wildlife and domestic animals.

Ethical animal research

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

Acknowledgements

The authors acknowledge Dr Peter Dommett for the provision of horses and Ms Megan Frost, management and staff at Waterford Farm Stud, KwaZulu-Natal Province for assistance in animal handling during data collection, Professor G.T. Fosgate for advice on manuscript preparation and Mr Ofentse Mogoba for technical laboratory assistance.

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.

References

[1] Ownby CL, Shivers CA. Antigens of the hamster ovary and effects of anti-ovary serum on eggs, Biol. Reprod. 1972; 6: 310-18

[2] Lyda RO, Hall JR, Kirkpatrick JF. A comparison of Freund's Complete and Freund's Modified Adjuvants used with a contraceptive vaccine in wild horses (Equus caballus), J Zoo Wildl Med 2005; 36(4): 610-16

[3] Liu IKM, Bernoco M, Feldman M. Contraception in mares heteroimmunized with pig zonae pellucida, J. Reprod. Fertil. 1989; 85: 19-29

[4] Kirkpatrick JF, Liu IKM, Turner JW. Remotely-delivered immunocontraception in feral horses, Wildl. Soc. Bull. 1990; 18: 326-30

[5] McShea WJ, Monfort SL, Hakim S, Kirkpatrick JF, Liu IKM, Turner JW, et al. Immunocontraceptive efficacy and the impact of contraception on the reproductive behaviours of white-tailed deer, J. Wildl. Manage. 1997; 61: 560-69

[6] Fayrer-Hosken RA, Grobler D, Van Altena JJ, Kirkpatrick JF, Bertschinger H. Immunocontraception of free-roaming African elephants, Nature 2000; 407: 149

[7] Bertschinger HJ, Delsink AK, Kirkpatrick JF, van Altena JJ, Ahlers M, Dickerson T, Powrie D, Burger A. Porcine zona pellucida immunocontraception of African elephants (*Loxodonta africana*): beyond the experimental stage. IVth International Wildlife Management Congress, James W. Cain III and Jason P. Marshal, Editors, 9-12 July 2012: 95-102

[8] Bertschinger HJ, Delsink A, van Altena JJ, Kirkpatrick JF. (2018). Porcine zona pellucida vaccine immunocontraception of African elephant (*Loxodonta africana*) cows: A review of 22 years of research, Bothalia - African Biodiversity & Conservation 2018; ISSN: (Online) 2311-9284, (Print) 0006-8241: 1-8

[9] Frank KM, Lyda RO, Kirkpatrick JF. Immunocontraception of captive exotic species. IV. Species differences in response to the porcine zona pellucida vaccine and the timing of booster inoculations, Zoo Biology 2005; 24:349-358

[10] Kirkpatrick JF, Turner A. Achieving population goals on long-lived wildlife with contraception, Wildl Res 2008; 35: 513-19

[11] Rutberg A, Naugle RE. Population-level effects of immunocontraception in white-tailed deer, Wildl Res 2008; 35: 494-501

[12] Delsink A, Van Altena JJ, Grobler D, Bertschinger H, Kirkpatrick JF, Slotow R. Implementing immunocontraception in free-ranging African elephants at Makalali conservancy, J S Afr Vet Assoc 2007; 78: 25-30

[13] Bechert U, Bartell J, Kutzler M, Menino A, Bildfell R, Anderson M, Fraker M. Effects of two porcine zona pellucida immunocontraceptive vaccines on ovarian activity in horses, The J Wildl Manag 2013; 77(7): 1386-1400

[14] Bechert U. The response of African elephants to a single dose of SpayVac®, a pZP contraceptive vaccine, over a seven-year period, Pachyderm 2016; 57: 97-108

[15] Barber MR, Fayrer-Hosken, RA. Possible mechanisms of mammalian immunocontraception, J Reprod Immunol 2000; 46: 103-24

[16] Joonè CJ, Bertschinger HJ, Gupta SK, Fosgate GT, Arukha AP, Munhas V, et al. Ovarian function and pregnancy outcome in pony mares following immunocontraception with native and recombinant porcine zona pellucida vaccines, Equine Vet J 2017; 49(2):189-95

[17] Ambrosia RL, Roberts BN, Roberts TA, DeYoung BL, Peterson EW, Bertschinger HJ, Schulman ML, Crampton M, Roth R, van Zyl PJ, Cameron-Blake N, Vandenplas ML, Knobel DL, French HM. Porcine and recombinant zona pellucida vaccines as immunocontraceptives for donkeys in the Caribbean, Clin Theriogenology 2017; 9(3): 439

[18] Kirkpatrick JF, Liu IMK, Turner JW, Naugle R, Keiper R. Long-term effects of porcine zonae pellucidae immunocontraception on ovarian function in feral horses (Equus caballus), J Repro Fertil 1992; 94(2): 437-44

[19] Apostólico J de S, Lunardelli VAS, Coirada FC, Boscardin SB, Rosa DS. Adjuvants: Classification, modus Operandi, and Licensing, J. Immunology Research, 2016; 6: 1–16. doi: 10.1155/2016/1459394

[20] Goto N, Akama K. Histopathological studies of reactions in mice injected with aluminium-adsorbed tetanus toxoid, Microbiology and Immunology 1982; 26(12): 1121-32

[21] Olive C. Pattern recognition receptors: sentinels in innate immunity and targets of new vaccine adjuvants, Expert Review of Vaccines 2012; 11(2): 237-56

[22] Lee S, Nguyen MT. Recent advances of vaccine adjuvants for infectious disease, Immune Network 2015; 15(2): 51-7.

[23] Gupta N, Chakrabarti K, Prakash K, Wadhwa N, Gupta T, Gupta SK. Immunogenicity and contraceptive efficacy of *Escherichia coli*- expressed recombinant porcine zona pellucida proteins, Am. J. Reprod. Immunol. 2013; 70: 139-52

480 [24] Mahi-Brown CA, Yanagimachi R, Hoffman JC, Huang TT Jr. Fertility control in the bitch
481 by active immunization with porcine zonae pellucidae: use of different adjuvants and
482 patterns of estradiol and progesterone levels in estrous cycles, Biol Reprod 1985;??

483 [25] Prasad SV, Skinner SM, Carino C, Wang N, Cartwright J, Dunbar BS. Structure and
484 Function of the Proteins of the Mammalian Zona Pellucida, Cells Tissues Organs 2000;
485 166(2): 148-164. doi: 10.1159/000016730

486 [26] Joonè CJ. Porcine zona pellucida immunocontraception in the mare: Clinical,
487 endocrinological and immunological effects, PhD Thesis 2016, University of Pretoria, South
488 Africa

489 [27] Joonè CJ, Schulman ML, Bertschinger HJ. Ovarian dysfunction associated with zona
490 pellucida–based immunocontraceptive vaccines, Theriogenology 2017; 89: 329-37
491 https://doi.org/10.1016/j.theriogenology.2016.09.018

492 [28] Billiau A, Matthys P. Modes of action of Freund's adjuvants in experimental models of
493 autoimmune diseases, J. Leukocyte Biol, 2001; 70(60): 849-60

494 [29] Kirkpatrick JF, Rowan A, Lamberski N, Wallace R, Frank K, Lyda R. The Practical Side
495 of Immunocontraception: Zona Proteins and Wildlife, J Reprod Immunol 2009, 83(1-2):151–
496 7. doi: 10.1016/j.jri.2009.06.257

497 [30] Gupta SK, Bansal P. 2010 Vaccines for immunological control of fertility, Reprod Med
498 Biol, 2010; 9: 61-71

499 [31] Henneke DR, Potter GD, Kreider JL. Relationship between condition score physical
500 measurements and body fat percentage in mares, Eq Vet J 1983; 15(4): 371-2

501 [32] Nolan MB, Bertschinger HJ, Roth R, Crampton M, Martins IS, Fosgate GT, Stout
502 TA, Schulman ML.Ovarian function following immunocontraceptive vaccination of mares
503 using native porcine and recombinant zona pellucida vaccines formulated with a non-
504 Freund's adjuvant and anti-GnRH vaccines**,** Theriogenology, 2018;120: 111-116

505 [33] Wassarman PM. Zona pellucida glycoproteins. Annu Rev Biochem 1988; 57:415–42.

506 [34] Safety and efficacy profile of Montanide™ Pet Gel A dedicated to sensitive veterinary
507 species. 2010. Technical bulletin. S.Deville Scientific marketing, Seppic, France

508 [35] Cauchard A, Bertrand F, Barrier-Battut I, Jacquet S, Laurentie M, Barbey C, et al.
509 Assessment of the safety and immunogenicity of Rhodococcus equi-secreted proteins
510 combined with either a liquid nanoparticle (IMS 3012) or a polymeric (Pet Gel A) water-
511 based adjuvant in adult horses and foals—Identification of promising new candidate
512 antigens, Vet Immunol Immunopathol 2014; 157: 164– 174

513 [36] Poly (I:C) (HMW) VacciGrade™ Polyinosinic-polycytidylic acid – TLR-3-based adjuvant,
514 Data sheet version # 11E06-MT, Invivogen, USA

515

Figure 1: Study 1 (geldings) mean anti-pZP antibody response expressed as a proportion of the positive standard (with 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)

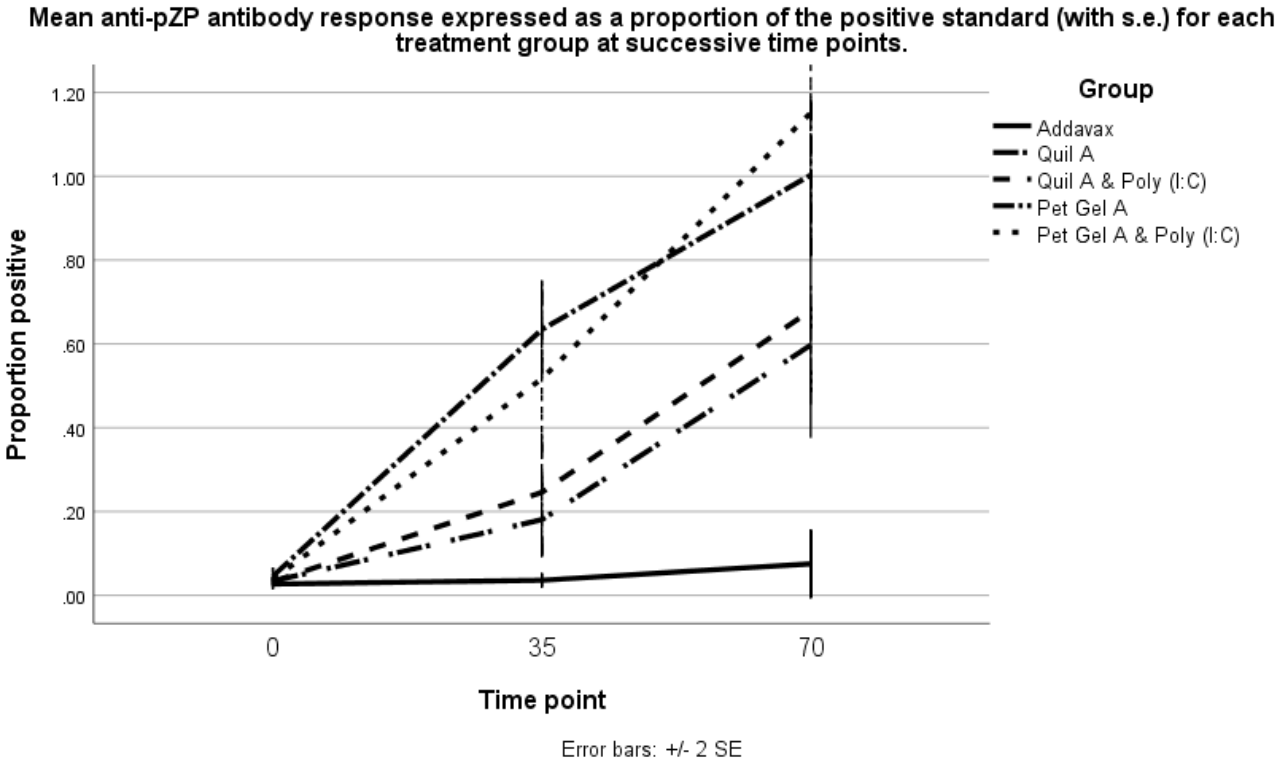


Figure 2: Study 2 (mares) mean anti-pZP antibody response expressed as a proportion of the positive standard (with s.e.) for all treatment groups (Adjuvant control: n=8; pZP only: n=7; reZP only: n=8; pZP & reZP: n=8) at successive time points 0 (d=0), 35 (d=35; reZP only), 70 (d=70), 105 (d=105) and 175 (d=175)

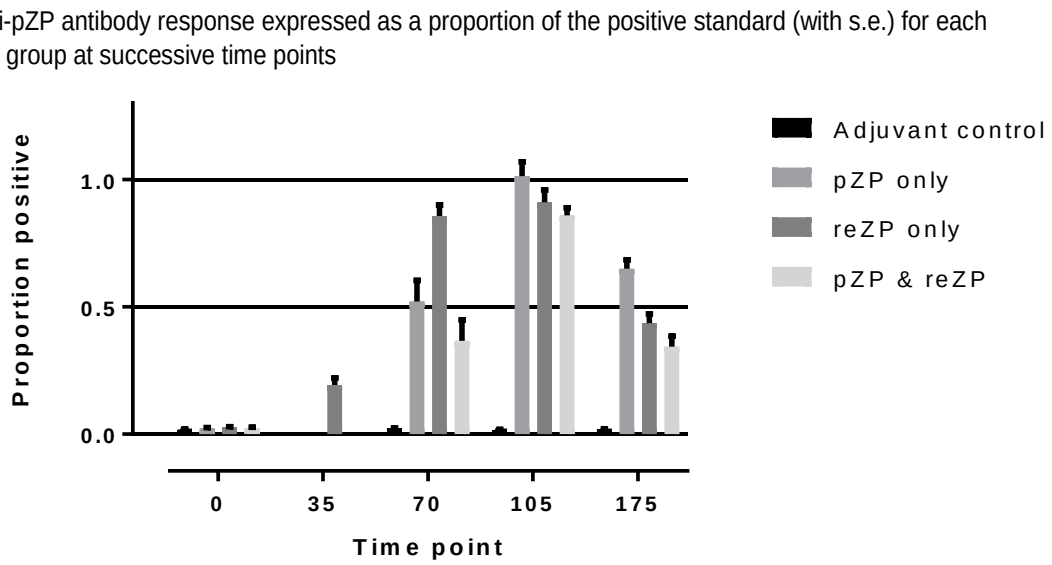
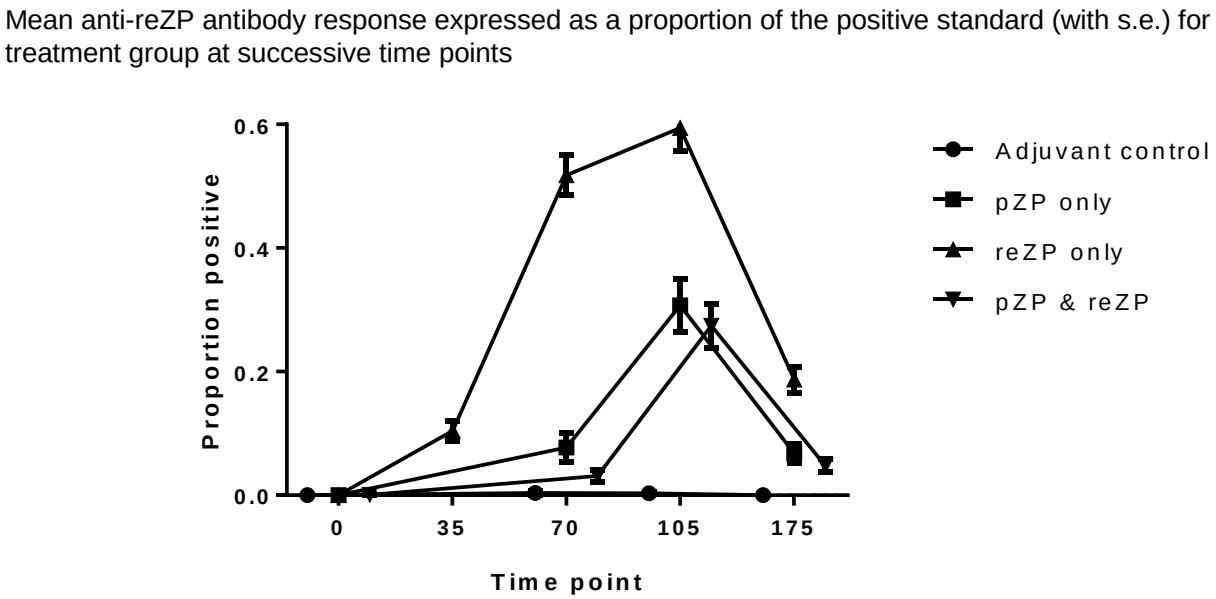


Figure 3: Study 2 (mares) mean anti-reZP antibody response expressed as a proportion of the positive standard (with s.e.) for all treatment groups (Adjuvant control: n=8; pZP only: n=7; reZP only: n=8; pZP & reZP only: n=8) at successive time points 0 (d=0), 35 (d=35; reZP only), 70 (d=70), 105 (d=105) and 175 (d=175)



532