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Phage display allows identification of zona pellucida-binding peptides with species-specific properties: Novel approach for development of contraceptive vaccines for wildlife

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ABSTRACT

Multiple phage-peptide constructs, where the peptides mimic sperm epitopes that bind to zona pellucida (ZP) proteins, were generated via selection from a phage display library using a novel approach. Selections were designed to allow for identification of ZP-binding phage clones with potential species-specific properties, an important feature for wildlife oral vaccines as the goal is to control overpopulation of a target species while not affecting non-target species' reproduction. Six phage-peptide antigens were injected intramuscularly into pigs and corresponding immune responses evaluated. Administration of the antigens into pigs stimulated production of anti-peptide antibodies, which were shown to act as anti-sperm antibodies. Potentially, such anti-sperm antibodies could interfere with sperm delivery or function in the male or female genital tract, leading to contraceptive effects. Staining of semen samples collected from different mammalian species, including pig, cat, dog, bull, and mouse, with anti-sera from pigs immunized with ZP-binding phage allowed identification of phage-peptide constructs with different levels of species specificity. Based on the intensity of the immune responses and specificity of these responses in different species, two of the antigens with fusion peptide sequences GEGGYGSHD and GQQGLNGDS were recognized as the most promising candidates for development of contraceptive vaccines for wild pigs.

1. Introduction

Immunocontraception is a promising strategy for wildlife population control. Similar to anti-disease vaccination, contraceptive vaccines stimulate the animal's own immune system to produce antibodies which interfere with one or more events in fertilization. While injectable immunocontraceptives were used successfully for many free-ranging mammalian species, including wild horses, urban deer, bison, and African elephants (Fagerstone et al., 2010; Kirkpatrick et al., 2011), they are not the most practical as parental immunization of each animal is required. To be economically viable, a contraceptive vaccine for wildlife species should be delivered orally. Oral vaccines must be species-specific as such preparations need to be distributed in uncontrolled environments and might contact non-target species, resulting in unwanted loss of fertility in the non-target animals.

Two reproductive antigens, porcine zona pellucida (PZP) and gonadotropin releasing hormone (GnRH), were used successfully for fertility control in wildlife; however, both lack species specificity. ZP is a glycoproteinaceous protective barrier, which surrounds each mammalian oocyte. It is essential for sperm–egg interaction and, therefore, conception. Preparations that use PZP proteins (either purified natural or recombinant proteins) as immunogens were shown to have contraceptive effects in multiple mammalian species (Fayrer-Hosken, 2008; Gupta et al., 2011). A second group of contraceptives uses immunogens based on GnRH, a master reproductive hormone. GnRH vaccines stimulate antibody production to inactivate endogenous GnRH that, in turn, causes reduced release of gonadotrophic hormones leading to gonadal atrophy. GnRH has been extensively studied for development of immunocontraceptive vaccines for various mammalian species,

Abbreviations: ZP, zona pellucida; PZP, porcine zona pellucida; GnRH, gonadotrophin releasing hormone; cfu, colony forming unit; vir, virion.

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including pigs (Killian et al., 2006), rodents (Jinshu et al., 2005), deer (Miller et al., 2008), cats (Levy et al., 2004), and dogs (Jung et al., 2005). However, immunocontraceptive vaccines that are derived from GnRH are not species-specific because GnRH is a ten amino acid-long peptide that is conserved in all mammalian species.

Sperm-specific antigens with cell surface expression that have a role in fertility (for example, fertilization antigen 1 (FA-1), sperm-specific lactate dehydrogenase C4, sperm antigen PH-20) also have been identified as potential targets for development of immunocontraceptive vaccines (Chamley and Clarke, 2007; Naz, 2011). Studies in this area are motivated by the fact that anti-sperm antibodies were recognized to be major factors in human infertility (Suri, 2005a,b). Our approach develops peptides mimicking sperm surface proteins that bind to ZP at fertilization. Immunization with such ZP-binding peptides stimulates production of anti-peptide antibodies, which might act as anti-sperm antibodies (Samoylova et al., 2010). Anti-sperm antibodies can reduce fertility by decreasing sperm motility, inhibiting the acrosome reaction, or interference with sperm–ZP binding, penetration, and fusion with the oocyte (Chamley and Clarke, 2007). Since sperm–ZP binding is suggested to occur via species-specific molecular recognitions (Nixon et al., 2007), antibodies interfering with sperm–ZP binding could prevent fertilization in a species-specific manner.

For identification of sperm antigens that participate in sperm–ZP binding, phage display technology can be utilized (Naz, 2005; Samoylova et al., 2010). The antigen identification by phage display includes several rounds of selection of ZP-binding phage from a phage display library, a multibillion mixture of genetically re-engineered phage clones. YLP_{12} peptide that bound to the human sperm–oocyte interactive site was selected via phage-display and used as an antigen for production of anti-sperm antibodies that prevented conception (Naz et al., 2000; Naz, 2004). Sperm–ZP binding is recognized as an event involved in determination of species-specific fertilization (Nixon et al., 2007); therefore, peptide antigens identified to bind to ZP have the potential to be species-specific.

As a target for identification of binding phage in this study, we used ZP-intact oocytes derived from pigs. Pigs were chosen as the target species because overpopulation of wild pigs is an expanding economic, health and welfare problem in many parts of the world (Hone, 2002; Massei and Genov, 2004; Mayer and Brisbin, 2009; West et al., 2009). The approach can potentially identify conserved as well as species-specific molecular interactions. To increase the chances of isolation of pig-specific ZP-binding phage, negative selection steps on ZPs from several mammalian species with close homology to pig ZP were added to the selection scheme. The selected phage clones displaying porcine ZP-binding peptides were used to immunize pigs for characterization of the corresponding immune responses. Antisera from these pigs were reacted with sperm from multiple mammalian species. These comparative experiments allowed us to determine which phage antigens stimulated the production of antibodies with the most specific recognition of pig spermatozoa. Such antigens represent candidates for development of species-specific contraceptive vaccines for wild pigs.

2. Materials and methods

2.1. Source of ovaries and isolation of ZP-intact oocytes

Ovaries were collected from the target species (pig) and three additional species (cat, dog, and cow) that were identified through a literature search to have the highest homology of ZP proteins (Conner et al., 2005; Mugnier et al., 2009). Canine and feline ovaries were collected from adult dogs and cats undergoing routine spays at a veterinary clinic in Georgia (Pet Vet Inc., Cumming, GA, USA). Porcine and bovine ovaries were obtained from a commercial source (PelFreez Biologicals, Rogers, AR, USA). Ovaries were frozen at −20 °C until required for experiments. Oocytes were isolated as previously described (Samoylova et al., 2010). Oocytes were washed in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) two times to remove cumulus cells, pooled and frozen (except pig oocytes) at −20 °C before further use. Pig oocytes were isolated as above one day before each phage selection experiment and kept on ice without freezing. Dark-field microscopy confirmed that the oocytes were surrounded by intact ZPs and no cumulus cells remained.

2.2. Semen collection

Boar semen was obtained from the Auburn University Swine Research and Education Center. Bull semen was obtained from the Auburn University Large Animal Hospital. Dog semen was collected by digital stimulation from healthy adult male beagle dogs at the Scott-Ritchey Research Center, Auburn University. Mouse semen was isolated from epididymis of euthanized mice by cutting the vas deferens into pieces and allowing the sperm to swim out into dishes with Embryomax (Millipore, Billierica, MA, USA) for 1 h at 37 °C and 5% CO_2. Cat testicles removed from adult animals at neuter surgeries were obtained from the Auburn University Small Animal Clinic. The epididymis was cut into several pieces and sperm allowed to swim out into PBS for 1 h at 37 °C and 5% CO_2. Semen samples with normal sperm motility (>70%) and no more than 10% abnormal spermatozoa were used for immunocytochemical studies. All protocols were approved by the Auburn University Institutional Animal Care and Use Committee.

2.3. Phage display library

To identify phage clones displaying peptides that bind to ZP on pig oocytes, a nonamer landscape phage display library was used (Kuzmicheva et al., 2009). The choice of a landscape library was motivated by studies showing that stronger immune responses to fusion peptides on phage were obtained with phage expressing higher peptide copy numbers (Yip et al., 2001; Wang and Yu, 2004). The landscape libraries are constructed in a phage vector type 8; therefore, foreign peptides on phage are displayed in each copy of the phage major coat protein VIII, resulting in a surface density of 4000 peptide copies per phage particle. High immunogenicity of peptides displayed on landscape phage was demonstrated previously (Minenkova et al., 1993).

All general methods of handling the landscape phage, including phage propagation, purification, titering, production of pure phage clones, and sequencing of phage DNA were performed as described by Brigati et al. (2008).

2.4. Selection of pig ZP-binding phage clones from a phage display library

First, an aliquot (3.5×10^9 cfu) of the primary library was diluted in 1 mL of a blocking buffer (0.1% BSA in PBS) and added to an empty polypropylene tube for overnight incubation to reduce the possibility of selection of false positive phage clones that bind to the plastic rather than to oocytes. To achieve species specificity, subtractive selection steps utilizing oocytes from three mammalian species other than pig were used prior to selections for ZP binding on pig oocytes. Buffer containing phage was transferred from the empty tube to a 1.5 mL tube with 1000 cat ZP-intact oocytes. After 1-h incubation at room temperature, the oocytes were pelleted by brief centrifugation (8000 rpm, 30 s) and the supernatant

(containing phage clones that did not bind to cat oocytes) was transferred to a tube with 1000 ZP-intact dog oocytes for similar incubation and separation. The same procedure was repeated once again on 1000 cow oocytes. Such subtractive selection steps were included in the selection scheme to remove phages that bind to ZPs of non-target species (cat, dog, and cow) as well as phage clones that are common for the target and non-target species. Finally, the supernatant with phage depleted on cat, dog, and cow oocytes was transferred to a tube with 1000 pig oocytes for one hour incubation. After that, phages not bound to oocytes were washed away with 0.1% BSA, 0.1% Tween 20 in PBS in repetitive (6–8 times total) centrifugation steps (8000 rpm, 30 s each). The oocyte pellet was lysed with 100 μL of a lysis buffer (2% deoxycholic acid sodium salt, 2 mM EDTA, 10 mM Tris–HCl, pH 8.0) to release bound phage. Phages recovered via lysis were used for amplification in *E. coli* K91BluKan host bacteria and in the subsequent round of selection for pig ZP binding (four rounds total). In the second, third, and fourth rounds, 1000 oocytes were incubated with 1×10^{10} cfu phage obtained from the previous selection round. To monitor the selection process, phage DNAs were amplified by PCR and sequenced after round two, three, and four.

2.5. Immunization of pigs with ZP-binding phage

Six phage clones selected for binding to pig ZP were propagated as individual phage preparations and purified from endotoxins as described elsewhere (Aida and Pabst, 1990). For pig immunizations, each phage antigen was formulated to include 1×10^{12} virions (vir) of phage in 250 μL PBS mixed with 250 μL of aluminum hydroxide adjuvant (Alhydrogel "85", Brenntag Biosector, Frederikssund, Denmark) and 100 μg immunostimulatory oligodeoxynucleotide (CpG, 5′-TCG TCG TTG TCG TTT TGT CGT T-3′) (Rankin et al., 2001; Yu et al., 2004) synthesized by Integrated DNA Technologies (Coralville, IA, USA). Phage concentration in virions per milliliter was assessed through reading the absorbance at 269 nm using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, Inc., Waltham, MA, USA). Phage preparations were injected intramuscularly into four-week old female piglets (three pigs/antigen). Booster immunizations at the same dosages were given at three and again at seven weeks following primary immunizations. Pre-immunization sera were used for corresponding controls. All animal use was approved by the Auburn University Institutional Animal Care and Use Committee.

2.6. Evaluation of pig sera for specific antibody responses by ELISA

Blood samples were collected from all pigs prior to immunization with phage antigens and monthly thereafter during a six-month period. Sera were separated from blood cells by one-step centrifugation (2000 g, 15 min). For detection of antigen-specific antibodies, phage clones used for pig immunization were diluted at 1.3×10^{12} vir/mL in TBS buffer and added to 96-well plates (80 μL/well) for overnight immobilization at 4 °C. After that, the supernatants were removed from the plates and 150 μL of 1% BSA in TBS were added to the wells for 1 h at 37 °C to block non-specific binding. Subsequently, the blocking solution was removed and wells were washed three times with TBS containing 1% BSA, 0.05% Tween-20. Serial two-fold dilutions of serum samples from immunized pigs were added to the phage-coated wells and the plates were incubated for 2 h at 37 °C. The wells were then washed three times with TBS containing 0.05% Tween-20. Goat anti-swine IgG (H + L) secondary antibody labeled with HRP (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:1000 in TBS containing 1% BSA and 0.05% Tween-20 was added to the wells for 1 h incubation at room temperature. The wells were washed as previously, reacted with o-phenylene diamine substrate solution, and absorbance read at 490 nm. To account for antibodies against phage coat proteins, serum samples were applied to wells coated with vector phage utilized for construction of the library, and processed in parallel with phage displaying fusion peptides. To calculate for specific antibody responses, OD numbers for the vector phage were subtracted from ODs obtained for recombinant phage. Each serum sample was evaluated in triplicate. Antibody responses were expressed as $\log_{10}$ of the end point titers (the highest sample dilution that produced an $OD_{490} \leq 0.1$).

2.7. Evaluation of pig sera for anti-sperm antibodies by immunocytochemistry

Semen samples collected from pig, dog, cat, bull, and mouse were washed with PBS buffer in two to three centrifugation steps at 500 g for 10 min at room temperature. The spermatozoa from all species were resuspended in PBS, sperm cell number counted, and adjusted to the concentration of 3×10^6 cells/mL. Thirty microliters of sperm cells were applied to Fisherbrand Superfrost/Plus microscope slides (ThermoFisher Scientific, Inc.) for five min immobilization at room temperature. After this, excess sperm cells were drained from the slides and the slides were dried at room temperature and then stored at 4 °C for up to two weeks. For detection of anti-sperm antibodies in pig serum samples, microscope slides with immobilized sperm cells were washed two times (5 min each) in TBS (50 mM/L Tris, 0.9% NaCl, pH 7.6). This was followed by 2 h incubation at room temperature in TBS containing 10% normal goat serum (Jackson ImmunoResearch Laboratories) and 1% BSA to block nonspecific binding. Samples of antisera with equal titers from pigs immunized with different antigens were diluted 1:2000 in TBS and applied to sperm cells. After 1 h incubation at room temperature, these slides were washed three times for 10 min in TBS, and then incubated for 1 h with 1:2000 dilution of FITC labeled goat anti-pig IgG (H + L) antibody (Jackson ImmunoResearch Laboratories) in TBS, 1% BSA. The slides were washed for 10 min with TBS three times, mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA), and examined by fluorescent microscopy. Pictures were taken using a Nikon Digital Sight camera and software (Nikon Instruments, Inc., Tokyo, Japan).

2.8. Statistics

Measurements of antibody titers were performed in triplicate and mean values are shown. Treatment groups were compared using one-way ANOVA at a significance value of $P \leq 0.05$ using Origin 7.5 data analysis and graphing software (OriginLab, Northampton, MA, USA).

3. Results

3.1. Identification of pig ZP-binding phage

To obtain phage clones with species specificity for pig ZP, negative selection steps on ZPs of different mammalian species were performed prior to selections on pig ZP (Fig. 1). Cat, dog, and cow ZPs were chosen for these subtractive (negative) selection steps because pig ZP proteins have close homology with ZPs of these species (Mugnier et al., 2009). The subtractive steps were included in the selection scheme to remove phage that bind to ZPs of non-target species as well as phage clones that are common for the target and non-target species. This was followed by three positive selection rounds on pig oocytes. Respectively, 39, 40, and 59 phage DNAs were sequenced after rounds two, three, and four, and ZP-binding peptides translated. Based on sequence similarities, the peptides were placed in two groups (Fig. 2). The presence of common and similar amino acids in specific positions (shaded

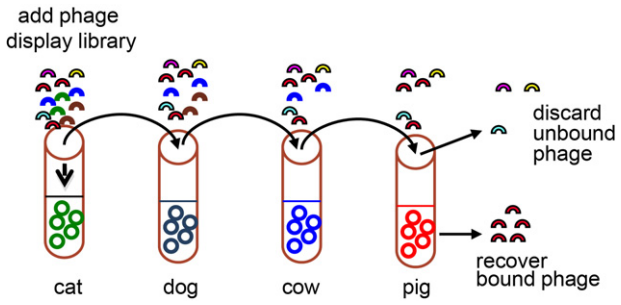

Fig. 1. Schematic representation of steps involved in selection of species-specific ZP-binding phage from a phage display library. To achieve porcine species specificity, negative selection steps utilizing ZP-intact oocytes from other mammalian species (cat, dog, and cow) are performed prior to selections on pig ZPs. First, the library is added to the tube containing ZP-intact oocytes of the first non-target species (cat). After incubation, non-binding phages are transferred to the tube containing ZP-intact oocytes of the second non-target species (dog). A third such incubation is with ZP-intact bovine oocytes. Following these subtractive steps, the remaining library is incubated with ZP-intact oocytes from the target species (pig). Finally, phages not bound to pig oocytes are removed by thorough washings and the oocytes are lysed to release bound phages. Phages recovered via lysis are amplified in bacteria and used in three additonal selection rounds on ZP-intact pig oocytes (not shown in the figure) to allow enrichment of phage clones specific for pig.

Group 1

GE GGY GS HD
GQQGL NGDS
GP NSS DADS

Group2

DV GGGT GT E
DP GL SL GDD
AY NLG E GDT

Fig. 2. Peptide sequences identified through selections from a 9-mer landscape phage display library for binding to ZP-intact porcine oocytes. The peptides are grouped based on their sequence similarities. Common and similar amino acids in specific positions are shaded.

in the figure) provides indirect evidence of specificity of the selection process. Combined frequency of peptides in group 1 was 35% after round two, and composed 20.1% and 28.8% in rounds three and four, respectively (see Table 1). Combined frequency of peptides in group 2 gradually increased with each additional round of selection (25% in round two, 48% in round three, and 59.4% in round four). Based on sequence analyses and round-to-round phage clone frequencies, six phage clones (three from each group) were chosen for immunogenicity tests in pigs.

3.2. Detection of specific antibodies against phage-displayed antigens in pig sera

ZP-binding phage clones identified as above were amplified, purified, and tested for immune responses in pigs in the whole phage particle format. Administration of phage into the pigs produced no visible adverse effects during the six-month trial period. Three pigs were immunized in each antigen group, and specific antibodies against phage-displayed antigens in pig sera were assayed by ELISA. As shown in Fig. 3, strong and consistent antibody responses were detected in serum of all animals. Although antibody responses varied in the first part of the experiment, no statistically significant differences were found among groups of pigs immunized with different antigens for the last three bleeding time points. Strong antibody responses in sera lasted at least six months (the duration of the experiment). At necropsy, oviducts of three pigs injected with antigen GEG were flushed with PBS, and the collected fluids were assayed for the presence of antigen-specific antibodies. The antibody titers in the collected samples were around 1:25,600. This was an important finding because oviducts are the site of fertilization. The presence of anti-sperm antibodies in this location might prevent sperm–ZP binding, leading to infertility.

Table 1
Frequencies of ZP-binding phage clones in subsequent selection rounds.

Antigen name	Peptide sequence	Frequency, %		
		Round 2	Round 3	Round 4
GEG	GEGGYGSHD	0	5.1	1.7
GPN	GPNSSDADS	15	5	6.8
GQQ	GQQGLNGDS	20	10	20.3
DVG	DVGGGTGTE	0	3	10.2
DPG	DPGLSLGDD	12.5	30	40.7
AYN	AYNLGEGDT	12.5	15	8.5

3.3. Evaluation of pig sera for presence of anti-sperm antibodies and their species specificity by immunocytochemistry

To further evaluate suitability of the phage antigens for development of contraceptive vaccines for wild pigs, the following two questions had to be answered: (1) whether antigen-specific antibodies detected in sera were anti-sperm antibodies, and (2) whether these antibodies were species-specific for the pig. To address these issues, semen samples were collected from multiple mammalian species, including pig, dog, cat, bull, and mouse, and reacted with sera from immunized pigs. It was found that all antigens indeed produced antibodies that reacted with pig sperm (Fig. 4, first column). The intensity of fluorescence on the pig sperm differed between the antigens in spite of the fact that antibody titers in sera used for the staining were similar. This might be explained by different numbers of binding sites specific for each antibody type and antibody affinities. Based on the antibody staining intensity on the pig sperm, the antigens can be placed in the following order GPN > GEG ≥ GQQ > DVG > DPG > AYN.

When tested on semen from multiple mammalian species, different antigens produced antibodies with different levels of species specificity (Fig. 4). Out of three antigens (GEG, GQQ, and GPN) with the strongest antibody binding to pig sperm, GEG and GQQ were found to be the most species-specific. No staining for GEG antigen was found on dog, cat, or mouse sperm. Specific staining was observed on bull sperm, but the intensity of the bull sperm staining was significantly lower than for pig, indicating that GEG antigen is a promising candidate for a species-specific vaccine for use in pigs. Antigen GQQ stimulated production of antibodies with good reactivity with pig sperm as well as some reactivity with dog sperm, but without binding to bull, cat, or mouse sperm. Antibodies stimulated by immunization with antigen GPN were less species-specific, demonstrating specific binding to several non-target sperm, including that of dog, bull, and mouse; however, the fluorescence was of significantly lower intensity than that on pig sperm. Pre-immunization sera diluted as post-immunization sera did not stain any of the semen samples, regardless of the antigen. Interestingly, none of the antigens stimulated production of antibodies which bind to cat sperm cells. GPN antibodies demonstrated some binding to small numbers (less than 30%) of mouse sperm cells.

Patterns of fluorescence depended on the origin of the spermatozoa. While at different intensities, fluorescent patterns of pig and bull sperm cells were similar. The staining was localized to the acrosomal region, with the most intense fluorescence at the anterior surface of the sperm head. Fluorescence on the dog sperm also was associated with the acrosome region, but was in a speckled pattern over the entire acrosomal surface. Unlike in other species, pattern of small dots were observed along sperm tails on mouse sperm stained with GPN serum.

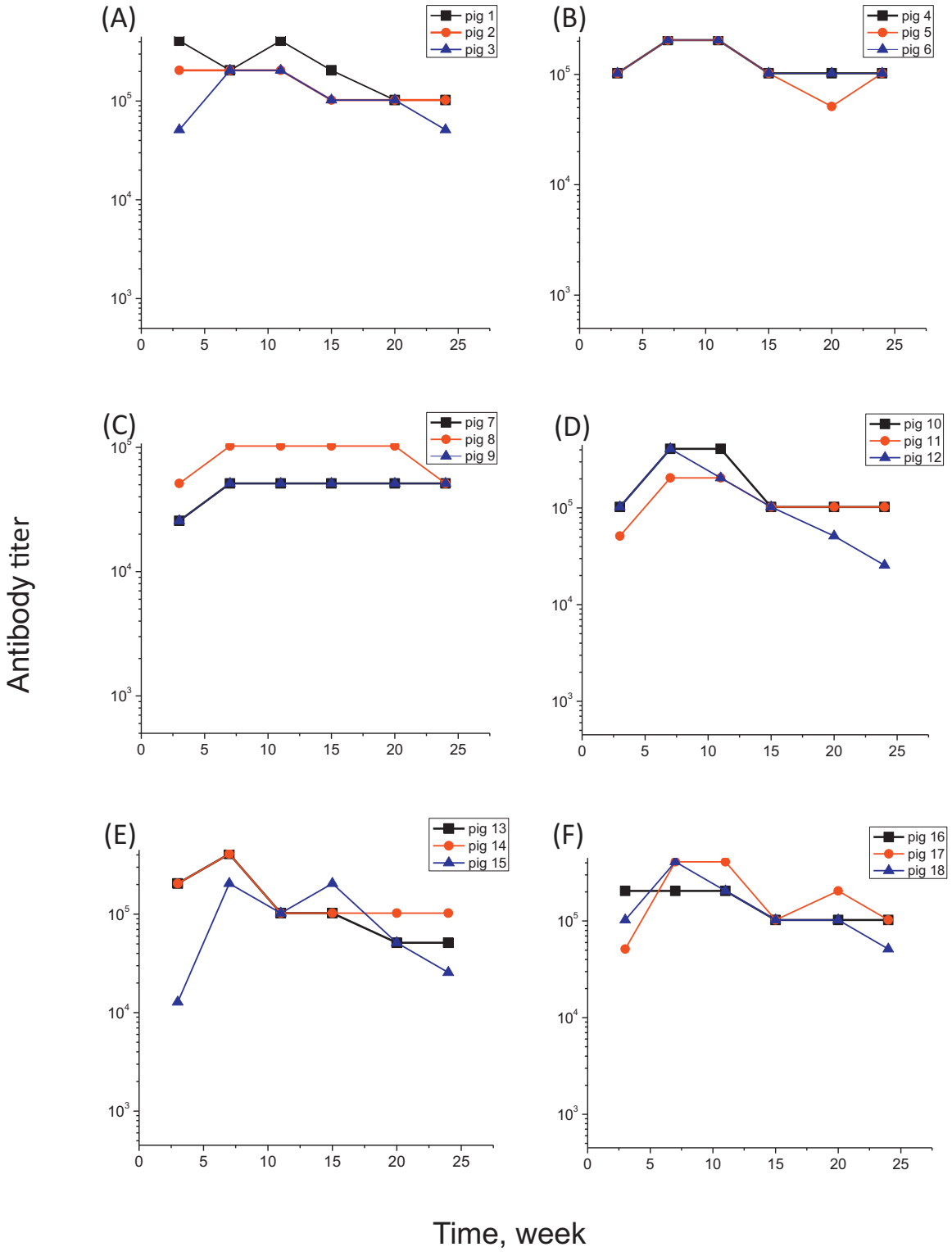

Fig. 3. Specific antibody responses in pigs immunized with six different ZP-binding phage antigens: (A) DVG; (B) GQQ; (C) DPG; (D) GPN; (E) AYN; (F) GEG. For complete peptide sequences see Table 1. Three pigs per antigen were immunized. Antibody titers (determined by ELISA) in serum samples collected from individual animals at six time points after immunization are shown. No appreciable ELISA responses were detected in sera samples collected before immunization. (*Note*: Data for three animals are shown for each antigen. In (B) and (C), partially overlapping data prevent visualization of separate curves.) Antibody responses are expressed as $\log_{10}$ of the end point titers.

4. Discussion

Current contraceptive vaccines for wild mammals are injectable and belong to two major groups, based on PZP or GnRH antigens, neither of which is species-specific. This represents a serious obstacle for the use of PZP or GnRH vaccines in oral formulations in uncontrolled field conditions since the vaccines can affect target as well as non-target animals. The third group of contraceptive vaccines (mostly studied for humans) uses various sperm proteins or their components that stimulate production of anti-sperm

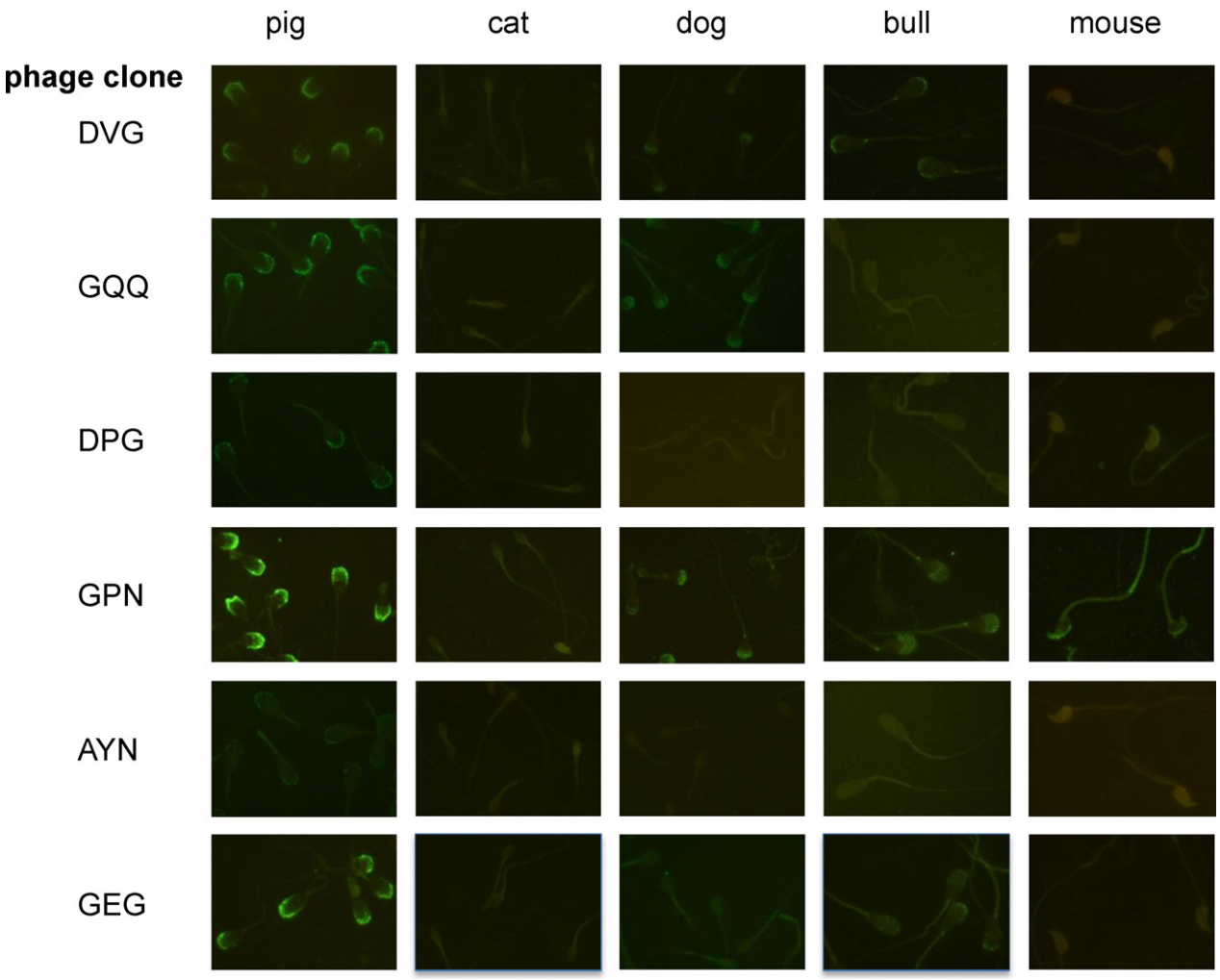

Fig. 4. Immunocytochemical staining of semen samples of different origin with antisera collected from pigs immunized with six different ZP-binding antigens. Animal species are shown horizontally. Phage-peptide antigens are shown vertically (for complete peptide sequences see Table 1). Semen samples collected from pig, dog, cat, bull, and mouse were adjusted to the concentration of 3×10^6 cells/mL and immobilized on microscope slides. For detection of anti-sperm antibodies, samples of antisera with equal titers from pigs immunized with different antigens were reacted with sperm cells followed by incubation with secondary FITC labeled goat anti-pig IgG antibody. Pre-immunization sera were used as controls to rule out non-specific binding. The figure is composed of representative pictures allowing comparison of serum reactivity against semen from different species as well as intensity of antiserum binding among antigens.

antibodies (Suri, 2005a), which might impede fertilization via decreasing sperm motility, inhibition of the acrosome reaction, or interference with sperm–oocyte interactions (Chamley and Clarke, 2007). Our approach develops phage-peptide constructs that bind to ZP through epitopes that mimic sperm proteins at fertilization. When administered into animals, such constructs could cause production of anti-sperm antibodies with contraceptive properties. We chose ZP as a target for identification of species-specific binding molecules since sperm–ZP interaction is proposed to be the major factor which defines species specificity of mammalian fertilization (Reid et al., 2011).

The precise mechanisms and the exact epitopes involved in sperm–ZP interactions remain to be clarified. It is known that ZP composition and structure varies among mammalian species. For example, unlike in other mammals, murine ZP comprises ZPA/ZP2, ZPC/ZP3, and ZP1 glycoproteins. ZP in the pig, dog, cat, cow, and rabbit comprises ZPA/ZP2, ZPB/ZP4, and ZPC/ZP3. Human, primate, rat, hamster, and horse ZPs are composed of ZPA/ZP2, ZPB/ZP4, ZPC/ZP3, and ZP1 (Goudet et al., 2008; Izquierdo-Rico et al., 2009; Mugnier et al., 2009). Sequence homologies between ZP proteins of different mammalian species (as compared to human ZP proteins) are rather high, ranging from 53 to 94.2% for ZP2, 48 to 93.9% for ZP3, and 55 to 92% for ZP4 (Choudhury et al., 2007), but none show complete homology. In addition to the protein components, sperm–ZP recognition is mediated by ZP glycosylation patterns, which might vary among species as well (Goudet et al., 2008).

To identify binders for porcine ZP, we used a phage display library of random peptides. This approach allows identification of binders that represent both conserved as well as species-specific molecular interactions. To improve chances of isolation of pig-specific phage binders, prior to reaction with porcine oocytes, the library was reacted with oocytes of non-target species (cat, dog, and cow) that have close homology to ZP proteins of the pig (Conner et al., 2005; Goudet et al., 2008). The peptide sequences isolated for binding to pig ZP were checked for sequence homologies to the proteins available through protein databases. While these searches did not reveal significant similarities with known sperm proteins, the isolated recombinant phage could resemble sperm cells structurally, acting as sperm cell mimetics. Further, six antigen preparations containing whole phage particles displaying pig ZP-binding peptides were injected into pigs. All of them stimulated production of anti-sperm antibodies; however, the level of their

species specificity significantly varied between antigens. For example, antibodies produced in response to GPN antigen (peptide group 1) recognized sperm of four (pig, bull, dog and mouse) out of the five tested species, while antigen GEG (also in peptide group 1) antibodies bound only to pig and bull sperm with the most binding seen on the pig sperm. Interestingly, the third antigen from peptide group 1, GQQ, stimulated antibodies with binding to pig and dog sperm. These results point to the fact that not only amino acids common for all three peptide antigens participate in phage-ZP recognition and binding, but it is likely that some of the flanking amino acids also contribute significantly to these events. Differences in staining between the species might suggest divergences in the molecular organization of their ZP glycoproteins.

While not the focus of this study, we evaluated pig weights as indirect parameters of pathologic change and examined reproductive tracts from the 18 immunized gilts at necropsy. No statistically significant differences in body weight among antigen groups at each time point were found. Reproductive tracts were normal on gross evaluation. No local or systemic adverse reactions were observed as a result of immunizations.

Whole phage particles displaying antigenic peptides were used as vaccines for different applications previously (Clark and March, 2006; Manoutcharian, 2011). Studies on development of phage-based vaccines explored preparations for treatment of melanoma (Eriksson et al., 2007, 2009), HIV (De Berardinis et al., 2003), Alzheimer's disease (Frenkel et al., 2003), candidiasis (Wang et al., 2006; Yang et al., 2007), and rabies (Houimel and Dellagi, 2009). Furthermore, recombinant phages displaying decapeptides of follicle-stimulating hormone receptor were shown to impair fertility in mice and inhibit ovulation rates in ewes (Abdennebi et al., 1999) and to induce infertility in adult male bonnet monkeys (Abdennebi et al., 1999; Rao et al., 2004), suggesting the potential use of phage-based vaccines for immunocontraception. Several studies indicated that vaccines based on phage have the potentials to be effective immunogens after oral administration (Delmastro et al., 1997; Jensen-jarolim et al., 1998; Zuercher et al., 2000). The demonstration of a protective effect of an oral phage vaccine against cysticercosis in pigs pointed toward the possibility for development of other oral vaccines for pigs based on phage (Manoutcharian et al., 2004). Additional tools such as pig-specific oral delivery systems (Campbell and Long, 2007; Campbell et al., 2011) or baits flavored with pig preferable attractants (Campbell and Long, 2009) might be used to increase pig species specificity.

To conclude, our study has developed a new approach for identification of species-specific molecules responsible for sperm–ZP interaction. The ability of the phage antigens studied here to stimulate production of antibodies which bind to sperm cells suggests that their administration might lead to contraceptive effects. Fertility studies in multiple species are necessary to demonstrate contraceptive potentials of the identified ZP-binding phage preparations as well as to confirm their species specificity in vivo. The novel technology outlined in this study can be applied to the control of overpopulations of multiple wildlife species.

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References

Abdennebi, L., Couture, L., Grebert, D., Pajot, E., Salesse, R., Remy, J.J., 1999. Generating FSH antagonists and agonists through immunization against FSH receptor N-terminal decapeptides. Journal of Molecular Endocrinology 22, 151–159.
Aida, Y., Pabst, M.J., 1990. Removal of endotoxin from protein solutions by phase-separation using triton X-114. Journal of Immunological Methods 132, 191–195.
Brigati, J.R., Samoylova, T.I., Jayanna, P.K., Petrenko, V.A., 2008. Phage display for Generating Peptide Reagents. In: Coligan, J.E., Dunn, B.M., Speicher, D.W., Wingfield, P.T. (Eds.), Current Protocols in Protein Science. John Wiley & Sons, Inc., USA, pp. 18.19.11–18.19.27.
Campbell, T.A., Long, D.B., 2007. Species-specific visitation and removal of baits for delivery of pharmaceuticals to feral swine. Journal of Wildlife Diseases 43, 485–491.
Campbell, T.A., Long, D.B., 2009. Strawberry-flavored baits for pharmaceutical delivery to feral swine. Journal of Wildlife Management 73, 615–619.
Campbell, T.A., Long, D.B., Massei, G., 2011. Efficacy of the Boar-Operated-System to deliver baits to feral swine. Preventive Veterinary Medicine 98, 243–249.
Chamley, L.W., Clarke, G.N., 2007. Antisperm antibodies and conception. Seminars in Immunopathology 29, 169–184.
Choudhury, S., Srivastava, N., Narwal, P.S., Rath, A., Jaiswal, S., Gupta, S.K., 2007. Feasibility and challenges in the development of immunocontraceptive vaccine based on zona pellucida glycoproteins. Society for Reproduction and Fertility 63, 479–493.
Clark, J.R., March, J.B., 2006. Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials. Trends in Biotechnology 24, 212–218.
Conner, S.J., Lefievre, L., Hughes, D.C., Barratt, C.L., 2005. Cracking the egg: increased complexity in the zona pellucida. Human Reproduction 20, 1148–1152.
De Berardinis, P., Sartorius, R., Caivano, A., Mascolo, D., Domingo, G.J., Del Pozzo, G., Gaubin, M., Perham, R.N., Piatier-Tonneau, D., Guardiola, J., 2003. Use of fusion proteins and procaryotic display systems for delivery of HIV-1 antigens: development of novel vaccines for HIV-1 infection. Current HIV Research 1, 441–446.
Delmastro, P., Meola, A., Monaci, P., Cortese, R., Galfre, G., 1997. Immunogenicity of filamentous phage displaying peptide mimotopes after oral administration. Vaccine 15, 1276–1285.
Eriksson, F., Culp, W.D., Massey, R., Egevad, L., Garland, D., Persson, M.A., Pisa, P., 2007. Tumor specific phage particles promote tumor regression in a mouse melanoma model. Cancer Immunology, Immunotherapy 56, 677–687.
Eriksson, F., Tsagozis, P., Lundberg, K., Parsa, R., Mangsbo, S.M., Persson, M.A., Harris, R.A., Pisa, P., 2009. Tumor-specific bacteriophages induce tumor destruction through activation of tumor-associated macrophages. Journal of Immunology 182, 3105–3111.
Fagerstone, K.A., Miller, L.A., Killian, G., Yoder, C.A., 2010. Review of issues concerning the use of reproductive inhibitors, with particular emphasis on resolving human-wildlife conflicts in North America. Integrative Zoology 5, 15–30.
Fayrer-Hosken, R., 2008. Controlling animal populations using anti-fertility vaccines. Reproduction in Domestic Animals 43 (Suppl. 2), 179–185.
Frenkel, D., Dewachter, I., Van Leuven, F., Solomon, B., 2003. Reduction of beta-amyloid plaques in brain of transgenic mouse model of Alzheimer's disease by EFRH-phage immunization. Vaccine 21, 1060–1065.
Goudet, G., Mugnier, S., Callebaut, I., Monget, P., 2008. Phylogenetic analysis and identification of pseudogenes reveal a progressive loss of zona pellucida genes during evolution of vertebrates. Biology of Reproduction 78, 796–806.
Gupta, S.K., Gupta, N., Suman, P., Choudhury, S., Prakash, K., Gupta, T., Sriraman, R., Nagendrakumar, S.B., Srinivasan, V.A., 2011. Zona pellucida-based contraceptive vaccines for human and animal utility. Journal of Reproductive Immunology 88, 240–246.
Hone, J., 2002. Feral pigs in the Namadgi national Park, Australia: dynamics, impacts and management. Biological Conservation 105, 231–242.
Houimel, M., Dellagi, K., 2009. Peptide mimotopes of rabies virus glycoprotein with immunogenic activity. Vaccine 27, 4648–4655.
Izquierdo-Rico, M.J., Jimenez-Movilla, M., Llop, E., Perez-Oliva, A.B., Ballesta, J., Gutierrez-Gallego, R., Jimenez-Cervantes, C., Aviles, M., 2009. Hamster zona pellucida is formed by four glycoproteins: ZP1, ZP2, ZP3, and ZP4. Journal of Proteome Research 8, 926–941.
Jensen-jarolim, E., Leitner, A., Kalchhauser, H., Zurcher, A., Ganglberger, E., Bohle, B., Scheiner, O., Boltz-nitulescu, G., Breiteneder, H., 1998. Peptide mimotopes displayed by phage inhibit antibody binding to bet v 1, the major birch pollen allergen, and induce specific IgG response in mice. FASEB Journal 12, 1635–1642.
Jinshu, X., Jingjing, L., Duan, P., Zheng, Z., Ding, M., Jie, W., Rongyue, C., Zhuoyi, H., Roque, R.S., 2005. A synthetic gonadotropin-releasing hormone (GnRH) vaccine for control of fertility and hormone dependent diseases without any adjuvant. Vaccine 23, 4834–4843.
Jung, M.J., Moon, Y.C., Cho, I.H., Yeh, J.Y., Kim, S.E., Chang, W.S., Park, S.Y., Song, C.S., Kim, H.Y., Park, K.K., McOrist, S., Choi, I.S., Lee, J.B., 2005. Induction of castration by immunization of male dogs with recombinant gonadotropin-releasing hormone (GnRH)-canine distemper virus (CDV) T helper cell epitope p35. Journal of Veterinary Science 6, 21–24.
Killian, G., Miller, L., Rhyan, J., Doten, H., 2006. Immunocontraception of Florida feral swine with a single-dose GnRH vaccine. American Journal of Reproductive Immunology 55, 378–384.
Kirkpatrick, J.F., Lyda, R.O., Frank, K.M., 2011. Contraceptive vaccines for wildlife: a review. American Journal of Reproductive Immunology 66, 40–50.
Kuzmicheva, G.A., Jayanna, P.K., Sorokulova, I.B., Petrenko, V.A., 2009. Diversity and censoring of landscape phage libraries. Protein Engineering Design and Selection 22, 9–18.

Levy, J.K., Miller, L.A., Cynda Crawford, P., Ritchey, J.W., Ross, M.K., Fagerstone, K.A., 2004. GnRH immunocontraception of male cats. Theriogenology 62, 1116–1130.
Manoutcharian, K., 2011. Phage vaccines and phage therapy. In: Petrenko, V.A., Smith, G.P. (Eds.), Phage Nanobiotechnology. RSC Publishing, London, pp. 245–258.
Manoutcharian, K., Diaz-Orea, A., Gevorkian, G., Fragoso, G., Acero, G., Gonzalez, E., De Aluja, A., Villalobos, N., Gomez-Conde, E., Sciutto, E., 2004. Recombinant bacteriophage-based multiepitope vaccine against Taenia solium pig cysticercosis. Veterinary Immunology and Immunopathology 99, 11–24.
Massei, G., Genov, P., 2004. The enviromental impact of wild boar. Galemys 16, 135–145.
Mayer, J.J., Brisbin Jr., I.L. (Eds.), 2009. Wild Pigs: Biology, Damage, Control Techniques, and Management. Savannah River Natioal Laboratory, Aiken, SC.
Miller, L.A., Gionfriddo, J.P., Fagerstone, K.A., Rhyan, J.C., Killian, G.J., 2008. The single-shot GnRH immunocontraceptive vaccine (GonaCon) in white-tailed deer: comparison of several GnRH preparations. American Journal of Reproductive Immunology 60, 214–223.
Minenkova, O.O., Ilyichev, A.A., Kishchenko, G.P., Petrenko, V.A., 1993. Design of specific immunogens using filamentous phage as the carrier. Gene 128, 85–88.
Mugnier, S., Dell'Aquila, M.E., Pelaez, J., Douet, C., Ambruosi, B., De Santis, T., Lacalandra, G.M., Lebos, C., Sizaret, P.Y., Delaleu, B., Monget, P., Mermillod, P., Magistrini, M., Meyers, S.A., Goudet, G., 2009. New insights into the mechanisms of fertilization: comparison of the fertilization steps, composition, and structure of the zona pellucida between horses and pigs. Biology of Reproduction 81, 856–870.
Naz, R.K., 2004. Human synthetic peptide vaccine for contraception targeting sperm. Archives of Andrology 50, 113–119.
Naz, R.K., 2005. Search for peptide sequences involved in human antisperm antibody-mediated male immunoinfertility by using phage display technology. Molecular Reproduction and Development 72, 25–30.
Naz, R.K., 2011. Antisperm contraceptive vaccines: where we are and where are we going? American Journal of Reproductive Immunology 66, 5–12.
Naz, R.K., Zhu, X., Kadam, A.L., 2000. Identification of human sperm peptide sequence involved in egg binding for immunocontraception. Biology of Reproduction 62, 318–324.
Nixon, B., Aitken, R.J., McLaughlin, E.A., 2007. New insights into the molecular mechanisms of sperm–egg interaction. Cellular and Molecular Life Science 64, 1805–1823.
Rankin, R., Pontarollo, R., Ioannou, X., Krieg, A.M., Hecker, R., Babiuk, L.A., van Drunen Littel-van den Hurk, S., 2001. CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. Antisense and Nucleic Acid Drug Development 11, 333–340.
Rao, A.J., Ramachandra, S.G., Ramesh, V., Couture, L., Abdennebi, L., Salesse, R., Remy, J.J., 2004. Induction of infertility in adult male bonnet monkeys by immunization with phage-expressed peptides of the extracellular domain of FSH receptor. Reproductive Biomedicine Online 8, 385–391.
Reid, A.T., Redgrove, K., Aitken, R.J., Nixon, B., 2011. Cellular mechanisms regulating sperm–zona pellucida interaction. Asian Journal of Andrology 13, 88–96.
Samoylova, T.I., Cox, N.R., Cochran, A.M., Samoylov, A.M., Griffin, B., Baker, H.J., 2010. ZP-binding peptides identified via phage display stimulate production of sperm antibodies in dogs. Animal Reproduction Science 120, 151–157.
Suri, A., 2005a. Contraceptive vaccines targeting sperm. Expert Opinion on Biological Therapy 5, 381–392.
Suri, A., 2005b. Sperm-based contraceptive vaccines: current status, merits and development. Expert Reviews in Molecular Medicine 7, 1–16.
Wang, G., Sun, M., Fang, J., Yang, Q., Tong, H., Wang, L., 2006. Protective immune responses against systemic candidiasis mediated by phage-displayed specific epitope of *Candida albicans* heat shock protein 90 in C57BL/6J mice. Vaccine 24, 6065–6073.
Wang, L.F., Yu, M., 2004. Epitope identification and discovery using phage display libraries: applications in vaccine development and diagnostics. Current Drug Targets 5, 1–15.
West, B.C., Cooper, A.L., Armstrong, J.B., 2009. Managing Wild Pigs: A Technical Guide. Berryman Institute, Starkville, MS and Logan, UT, USA.
Yang, Q., Su, Q.P., Wang, G.Y., Wen, D.Z., Zhang, Y.H., Bao, H.Z., Wang, L., 2007. Production of hybrid phage displaying secreted aspartyl proteinase epitope of *Candida albicans* and its application for the diagnosis of disseminated candidiasis. Mycoses 50, 165–171.
Yip, Y.L., Smith, G., Ward, R.L., 2001. Comparison of phage pIII, pVIII and GST as carrier proteins for peptide immunisation in Balb/c mice. Immunology Letters 79, 197–202.
Yu, H., Babiuk, L.A., van Drunen Littel-van den Hurk, S., 2004. Priming with CpG-enriched plasmid and boosting with protein formulated with CpG oligodeoxynucleotides and Quil A induces strong cellular and humoral immune responses to hepatitis C virus NS3. Journal of General Virology 85, 1533–1543.
Zuercher, A.W., Miescher, S.M., Vogel, M., Rudolf, M.P., Stadler, M.B., Stadler, B.M., 2000. Oral anti-IgE immunization with epitope-displaying phage. European Journal of Immunology 30, 128–135.