

## Partial Characterization of Antigenic Sperm Proteins in Foxes (*Vulpes vulpes*)

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**ABSTRACT:** The aim of this work was to identify antigenic proteins on fox spermatozoa. Fox spermatozoa proteins were injected into 3 female rabbits and into 3 male and 3 female foxes. In rabbits, a rapid humoral response was observed. Using rabbit sera for Western blotting, 23 fox sperm protein bands were recognized between 10 and 110 kd. In foxes, the time course of antibody response was studied in the same manner. The number of recognized bands was maximal on day 75 for 2 foxes, on day 90 for 3 foxes, and on day 120 for 1 fox. Western blot patterns varied from one fox to another. On the whole, 25 protein bands between 10 and 110 kd were recognized. Using fluorescein isothiocyanate (FITC) labeling on fox spermatozoa

with rabbit and fox sera, we showed that several antigens recognized by the antisera were located at or near the surface of the spermatozoa. By two-dimensional electrophoresis and gel-purification, we have selected 6 highly antigenic proteins with molecular weights of 11.4, 14.7, 16.4, 16.4, 16.8, and 16.9 kd, and isoelectric points of 6.0, 6.0, 6.2, 5.5, 5.3, and 5.8, respectively, and one antigenic protein at 97 kd with an isoelectric point of 4.3 to 4.6. The results of this study can be used to characterize these 7 antigens selected more precisely by microsequencing or mass spectrometry.

Key words: Antisperm antibody, fox, sperm antigen, spermatozoa.  
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Identification of sperm antigens has become the object of increasing attention as a means of furthering our understanding of molecules involved in the fertilization process and for discovering potential targets for antisperm contraceptive vaccines. For the latter purpose, the identification of sperm proteins would be necessary for each studied species. Indeed, interspecies differences have been made apparent by molecular analysis of sperm proteins that appear to mediate the same events, and are also demonstrated by the diversity of sperm morphology as well as by the inefficiency of heterospecific fertilization (McLeskey et al, 1998).

In the fox (*Vulpes vulpes*), only a few sperm proteins have as yet been described: FSA-1 (Beaton et al, 1994), LDH-C4 (Bradley et al, 1996) and PH-20 (ten Have et al, 1998). Moreover, because these sperm proteins are not specific to the fox, the use of these antigens for oral vaccination could affect other wild populations. Furthermore, the use of these proteins for immunocontraception in female foxes have not given results that are as good as those in others species (Bradley et al, 1997; De Jersey et al, 1999). A cocktail composed of several antigens is probably required to achieve a high efficiency of immunocon-

trapection. Consequently, it could be necessary to identify more fox sperm antigens.

The immunological approach has been used with success to identify sperm proteins (Haden et al, 2000). Sperm-specific proteins are able to induce immune responses in males because they are synthesized and incorporated into spermatozoa only at puberty, long after the neonatal period, during which the immune system sorts “self” from “non-self” (Tung, 1998). By this age, the contact between sperm antigens and the immune system is restricted, as the male germ cells are sequestered behind the blood-testis barrier during last stages of their development (Pelletier, 1998). Thus, by immunization with sperm proteins, it is possible to induce a humoral response against sperm-specific antigens.

The aim of this work was to identify sperm antigens for an immunocontraceptive application in the fox population for both sexes. Using an immunological approach, we have selected 7 highly antigenic proteins. The results obtained can be used to sequence these highly antigenic fox sperm antigens by mass spectrometry or Edman chemistry.

### Materials and Methods

#### Chemicals

All chemicals were provided by Sigma Chemical Company, St Louis, Mo, except where specifically stated.

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### Animals

For the production of antibodies (protocol 1), we used 3 female New Zealand white rabbits, 12 weeks old, identified as R1–R3, and obtained from the Elevage Scientifique des Dombes (Romans, France).

For the immunization procedures (protocol 2), we used 6 healthy 2-year-old foxes (*Vulpes vulpes*) purchased from the Norwegian Fur Breeders Association (Eidsvoll, Norway). They were housed individually in outdoor cages in compliance with the Canadian Council on Animal Care guidelines in our experimental farm (agreement A 54747). The foxes were fed a daily ration of 120 g of dog food (27 g protein, 16 g fat, and 37 g carbohydrate per 100 g of food), and were provided with water ad libitum.

For the sperm protein extraction, 50 wild foxes were used. The animals had been caught for rabies monitoring during the reproduction period in northeastern France (January to March).

### Sperm Protein Extraction

Sperm samples were obtained from wild foxes. Epididymides were dissected and sperm was collected from the cauda epididymis by retrograde flushing from the vas deferens toward the proximal cauda with phosphate-buffered saline (PBS; 140 mM NaCl, 16 mM KCl, 6.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, and 1 mM MgCl<sub>2</sub> pH 7.3). Spermatozoa samples were pooled and washed twice by centrifugation (5 minutes at 800 × g at room temperature) with PBS, and pellets of 10<sup>8</sup> spermatozoa were stored at –80°C until analyzed (maximum 9 months).

For the one-dimensional Western blots, sperm proteins were extracted from each frozen sperm pellet by solubilization in 200 µL of sample buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol in bidistilled water). For the two-dimensional Western blots, each pellet was solubilized in 200 µL of a solution of 9.5 M urea, 2% Igepal, 2% 3–10 Ampholines (Pharmacia Biotech, Orsay, France), and 5% β-mercaptoethanol in twice distilled water.

For the immunization procedures, sperm proteins were extracted directly from washed fresh sperm (without freezing) with 100 µL/pellet of one of the following detergent solutions: solution A, 0.1% (v/v) Triton 100; solution B, 30 mM *n*-Octyl-β-D-glucoside; or solution C, 0.05 % (v/v) Igepal. Into each solution (A, B, and C) we added 0.15 mM aprotinin, 0.2 mM equine chorionic gonadotropin, and 0.1 mM benzamidine in PBS.

After agitation on a rotator for 5 minutes at room temperature (22°C), the samples were centrifuged (5 minute, 14 000 × g). The pellet was discarded and the protein concentration in the supernatant was evaluated using a colorimetric method, D<sub>C</sub> Protein Assay (Bio-Rad, Hercules, Cal), according to the supplier's instructions, using bovine serum albumin (BSA; fraction V) as the standard.

### Immunization Procedures

*Protocol 1*—Two-hundred micrograms of proteins extracted with solutions A, B, or C were emulsified with complete Freund adjuvant (CFA) and injected subcutaneously into rabbits R1, R2, and R3, respectively. After 3 weeks, a booster injection of the same protein preparation (stored at –20°C), emulsified in incom-

plete Freund adjuvant (IcFA) was given. Sera were collected 3 weeks later on day 42.

*Protocol 2*—Three-hundred micrograms of proteins extracted with solution A, B, or C were emulsified with CFA and injected subcutaneously into 3 male (F1, F3, and F5) and 3 female (F2, F4, and F6) foxes at the beginning of the breeding period (300 µg/fox). Two booster injections of 300 µg of proteins emulsified in IcFA were given subcutaneously at 1-month intervals. Sera were collected on days 0, 60, 75, 90, and 120.

### Immunolocalization of Antigens Recognized by the Antisera Using FITC Labeling

Smears of washed fox spermatozoa were prepared on glass slides, air-dried, and fixed for 5 minutes with cold methanol. Slides were then covered with PBS containing 1% BSA for 45 minutes to block nonspecific antibody binding. They were then incubated at room temperature in a humidified chamber for 2 hours with rabbit or fox sera diluted to 1:200, or with a control fox sera (1:200). For the fox sera, slides were then washed 3 times in PBS, and a 1:500 dilution of a rabbit anti-dog immunoglobulin (Ig) G antibody (Nordik) was added for 1 hour. All slides were then washed and incubated for 1 hour with goat anti-rabbit IgG-FITC-conjugated antibody (Sigma) diluted to 1:100. After 3 washings, slides were mounted with PBS-glycerol (1:1 v/v) and observed on an epifluorescent microscope (Olympus Optical Company, Hamburg, Germany). Photographs were obtained with a digital camera (DP50, Olympus).

### Electrophoresis and Western Blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions according to the methods of Laemmli (1970).

The two-dimensional gel electrophoresis was based on the procedure of O'Farrell (1975). Sperm proteins were run on a first-dimensional 4% urea gel pre-equilibrated with 9.5 M urea and a pH of 3–10 ampholines. To evaluate the isoelectric point (IP) as a function of the migration distance, 2 gels were cut into 5-mm-wide bands; each band was subjected to ultrasound for 5 minutes in distilled, outgassed water; and the pH of each solution was measured. The second-dimension electrophoresis was performed in a 12% acrylamide gel at 12°C.

Gels were then stained with Coomassie blue according to the method of Wu and Welsh (1996), or used for Western blotting. For the latter, sperm proteins were transferred onto nitrocellulose membrane according to the method of Towbin et al (1979). The blots were saturated with 5% (w/v) defatted milk (Bio-Rad) in PBS for 1 hour, washed for 5 minutes in PBS, and incubated overnight in the presence of fox sera (1:1000 diluted in PBS). The membranes were then washed 3 times with PBS-Tween and incubated for 1 hour with a rabbit anti-dog IgG (Nordic, Tilburg, The Netherlands) diluted to 1:1000 in PBS. After 3 washings with PBS-Tween, membranes were incubated for 1 hour with peroxidase-conjugated anti-rabbit IgG at 1:30 000 (Sigma) and washed 3 times with PBS-Tween. The immune complexes were detected with a chemiluminescent substrate of the peroxidase (SuperSignal; Pierce, Rockford, Ill) according to the supplier's instructions.

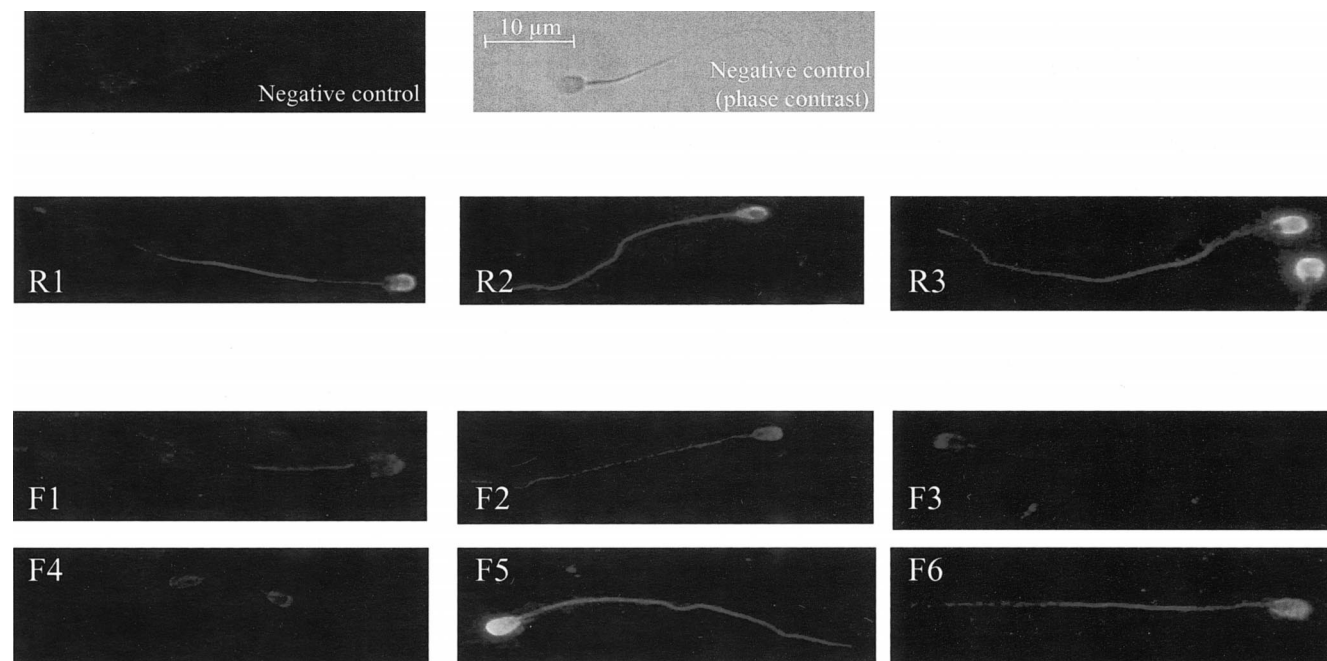


Figure 1. Immunofluorescence staining and phase contrast of fox sperm preparation by the sera of a control fox, of the rabbits R1 to R3 taken on day 42, and of the foxes F1 to F6 taken on day 90. Final magnification is 400 $\times$ .

### *Selection of the Antigenic Proteins and Determination of Their Molecular Weight*

With two-dimensional Western blotting, we determined 2 main areas containing antigenic proteins, but further investigations were necessary to determine precisely which proteins from these areas were antigenic. Thus, each protein purified by two-dimensional electrophoresis was tested individually by one-dimensional Western blot.

Spots of each protein visualized with Coomassie blue staining in the areas of interest were mashed individually in the sample buffer solution, and heated for 20 minutes at 56°C and for 5 minutes at 95°C before a new migration on a SDS-PAGE. To select the antigenic proteins, 10 spots of each protein were pooled (to increase the sensitivity of the method) and then transferred onto nitrocellulose membranes. Membranes were cut into bands, which were then incubated with different sera of rabbits and foxes. Immunoblot complexes were revealed by Western blot, as described in the "Electrophoresis and Western Blotting" section.

For an accurate determination of the molecular weight, 3 pooled spots of each protein were migrated on a Tris-Tricine gel (Bio-Rad), according to the supplier's instructions, and stained with silver nitrate according to the method of Morrissey (1981).

## **Results**

### *Localization of Recognized Antigens on the Spermatozoa Surface*

Figure 1 shows antigens localized at or near the surface of fox spermatozoa recognized by sera of rabbits R1–R3

and foxes F1–F6, visualized by indirect immunofluorescence staining. Comparison with phase contrast images indicated that for all samples, more than 90% of the spermatozoa were stained (data not shown). A control serum sample showed no significant staining.

In the rabbit sera, we observed 2 patterns of sperm staining: 1) an intense signal both on the entire head and on the principal piece, with a signal of lesser intensity on the mid piece (R1); and 2) an intense signal on the head, the mid piece, and the principal piece (R2 and R3).

In the fox sera, we observed 4 patterns of sperm staining: 1) a signal of medium intensity on both the entire head and the mid piece (F1 and F3); 2) a signal of medium intensity on the head, the mid piece, and the principal piece (F2 and F6); 3) a signal of weak intensity only on the head (F4); and 4) an intense signal on the head, with a signal of less intensity on the mid piece and the principal piece (F5).

### *Humoral Response to Sperm Protein Immunization*

To determine which specific antigens were recognized by antisperm antibodies after immunization, sera were studied by Western blot analysis.

In rabbits (protocol 1), immunization with fox spermatozoa proteins induced a rapid humoral response. Indeed, after 2 injections, on day 42, rabbit sera recognized all 23 different fox sperm antigens, the molecular weights of which varied between 10 and at least 110 kd (Figure 2). Among these bands, 10 were recognized by at least 2 rabbits, regardless of the protein extract injected. These

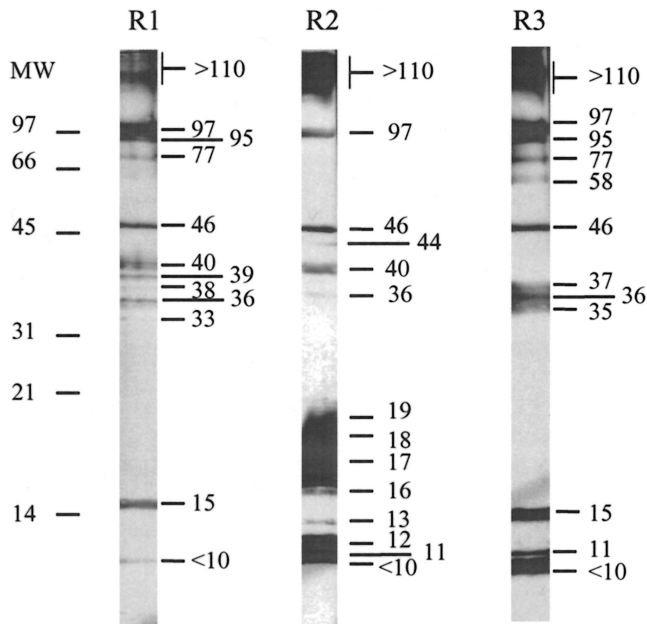


Figure 2. Western blots of fox sperm extract probed with rabbit sera. Molecular weights of the standards (kd) are indicated on the left, and molecular weights of the recognized bands are indicated on the right. On day 42, the rabbit sera recognized 23 different fox sperm antigens, the molecular weights of which vary between 10 and  $\geq 110$  kd.

proteins have a molecular weight of more than 100, 97, 95, 77, 46, 40, 36, 15, 11, and more than 10 kd.

In the foxes, all males and females responded to fox sperm protein immunization with the production of anti-sperm antibodies. Considering the number of recognized proteins, no significant difference was observed between the males and the females, regardless of the day considered (Figure 3). The number of recognized bands increased after each sperm extract injection and reached a maximum on day 75 for 2 foxes (F4 and F5), on day 90 for 3 foxes (F1, F2, and F6), and on day 120 for 1 fox (F3). The maximal numbers of protein bands recognized by the male and female fox sera were, respectively,  $8.6 \pm 3.0$  and  $6.6 \pm 2.1$ .

The molecular weights of the 25 bands recognized by 1 or more fox sera on day 90 (foxes F1, F2, F5, and F6) or on day 120 (F3 and F4) ranged from 10 to  $\geq 110$  kd (Figure 4). Of those bands, fox sera frequently recognized protein bands of molecular weights less than 30 kd, particularly proteins between 10 and 20 kd, and one band at 97 kd.

#### Localization on Two-Dimensional Gel Electrophoresis

After two-dimensional gel electrophoresis (Figure 5A), Western blot with rabbit (protocol 1) and fox (protocol 2) sera were realized. Two areas with highly antigenic proteins were determined.

The first area contained one protein of 97 kd with an IP of 4.3 to 4.6. This protein was recognized by the sera

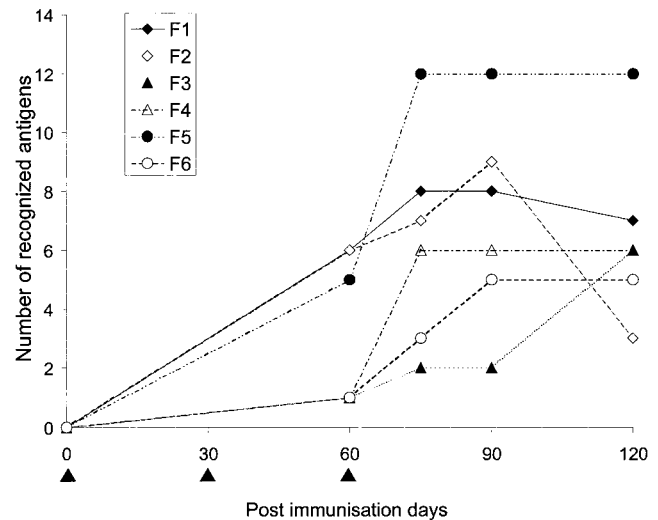


Figure 3. Number of sperm proteins recognized by SDS-PAGE Western blot with sera of male (F1, F3, and F5) and female (F2, F4, and F6) immunized foxes.  $\square$  Indicates days of immunization.

of the 3 rabbits and by the sera of 3 out of the 6 foxes from the protocol 2 (Figure 5B).

The second area contained proteins of low molecular weight, between 9 and 20 kd, and with an IP of 4.9 to 6.8. After Coomassie blue staining of the acrylamide gel, 12 proteins were visible (Figure 5A). The sera of the 3 rabbits and of 5 out of the 6 foxes recognized 1 or more proteins in this area (Figure 5C).

To determine which proteins were antigenic in these areas, the 12 proteins of low molecular weight (named P1 to P12) and 1 protein with a molecular weight of 97 kd (P13) were gel-purified and tested by Western blot, with sera of the 3 rabbits (protocol 1) and of the 6 foxes (protocol 2). The results are given in Table 1.

Out of the 12 proteins of low molecular weight, 5 were not recognized by any of the sera (P1, P2, P9, P10, or P12) and 1 serum, P6, was recognized only by 1 serum (rabbit R2). Three proteins were recognized by some sera (P3, P5, and P7). Three other proteins were recognized by a larger number of sera from the 2 experiments (P4, P8, and P11). The intensity of the reaction was high for some sera with these 3 proteins (Figure 6). The accurate molecular weights of these proteins, determined with Tris-Tricine gels, are between 11.4 and 16.9 (Table 2).

The protein P13 was recognized by the 3 rabbit sera, as well as by 3 out of the 6 fox sera tested, and the intensity of the reaction was high (Table 1 and Figure 6).

## Discussion

In this study we investigated the characteristics of the humoral response after immunization with fox spermatozoa proteins in order to purify fox sperm antigens. On

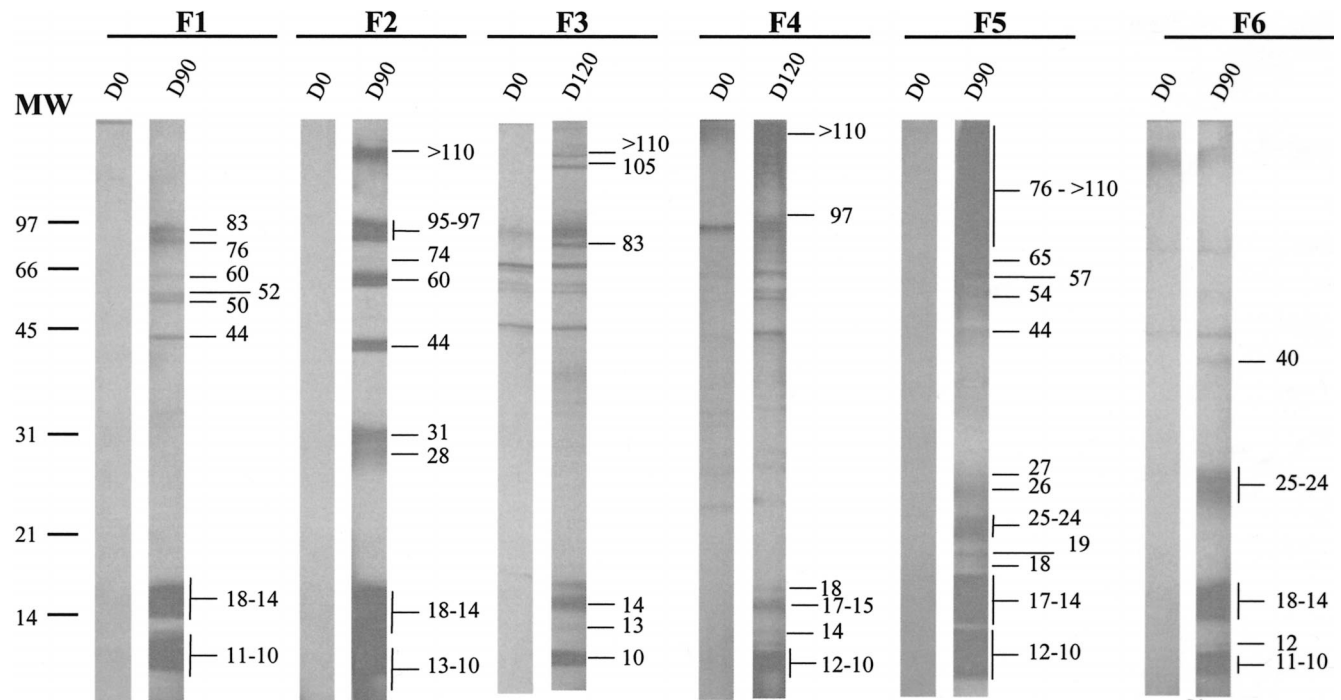


Figure 4. Western blots of fox sperm extract probed with fox sera taken on day 0 and on day 90 (F1, F2, F5, and F6) or day 120 (F3 and F4). Molecular weight standards ( $\text{kd} \times 10^{-3}$ ) are indicated on the left, and molecular weights of the recognized bands are indicated on the right. The molecular weights of the 25 recognized proteins bands ranged from 10 to  $>110$  kd.

the basis of these data, we selected the most antigenic proteins, which were then characterized by electrophoresis.

We showed that, independent of the extraction procedure used, it is possible to obtain antibodies against fox sperm proteins. To extract these proteins, Triton X-100, Igepal and *n*-Octyl- $\beta$ -D-glucoside were used as detergents. It has been shown that spermatozoa demembrated with the Triton X-100 could be reactivated in some pH conditions (Giroux-Widemann et al, 1991). This finding implies that low concentrations of this detergent do not affect the intracellular proteins, but they could extract the membrane and the associated proteins. Other detergents have been used successfully to extract membrane sperm antigens, for instance: SOB2 with Igepal (Lefèvre et al, 1997), and P34H with *n*-Octyl- $\beta$ -D-glucoside (Boué et al, 1996). From a methodological point of view, it should be noted that in our study it was necessary to freeze the fox spermatozoa, because foxes have one breeding season per year and produce sperm only 3 months per year. The freezing of cells before extraction probably destroyed membranes and many organelles. Therefore, it is probable that the sera could cross-react with both surface and intracellular proteins by Western blot. Nonetheless, FITC labeling confirms the presence of antigens either located on the sperm surface or exposed during air-drying and methanol fixation. Moreover, the diversity of the patterns obtained indicates that antisera

recognized several sperm antigens. It should be noted that these proteins were purified from epididymal spermatozoa, so their origin is testicular or epididymal.

After immunization with fox surface-sperm proteins, the humoral response of rabbits was characterized by a rapid production of antibodies, a relatively small number of recognized proteins, and a good homogeneity in the pattern of antigens. Forty-two days after the immunization, the rabbit sera recognized on average  $12.6 \pm 1.2$  bands by Western blot. This number is close to the number of alloantigens obtained (14 to 18) after immunization of gilts with pig membrane sperm protein (Haden et al, 2000), and superior to the number of bands (4 to 9) obtained after immunization of rabbits with sperm isoantigens extracted with several detergents (Naz et al, 1984). Although different detergents were used, it is probable that sperm heteroantigens are more numerous than isoantigens. Ten out of the 23 bands stained by Western blot were recognized by at least 2 rabbit sera out of the 3. This makes it probable that there is only a relatively small set of sperm heteroantigens that can be regarded as highly antigenic.

The humoral response of the foxes immunized with isoantigens differs from that of the rabbits: the antibody production is less rapid, fewer proteins are recognized by the sera, and the humoral response is more heterogeneous. After immunization, the kinetics of the humoral serum response show a temporary appearance of antibodies; the

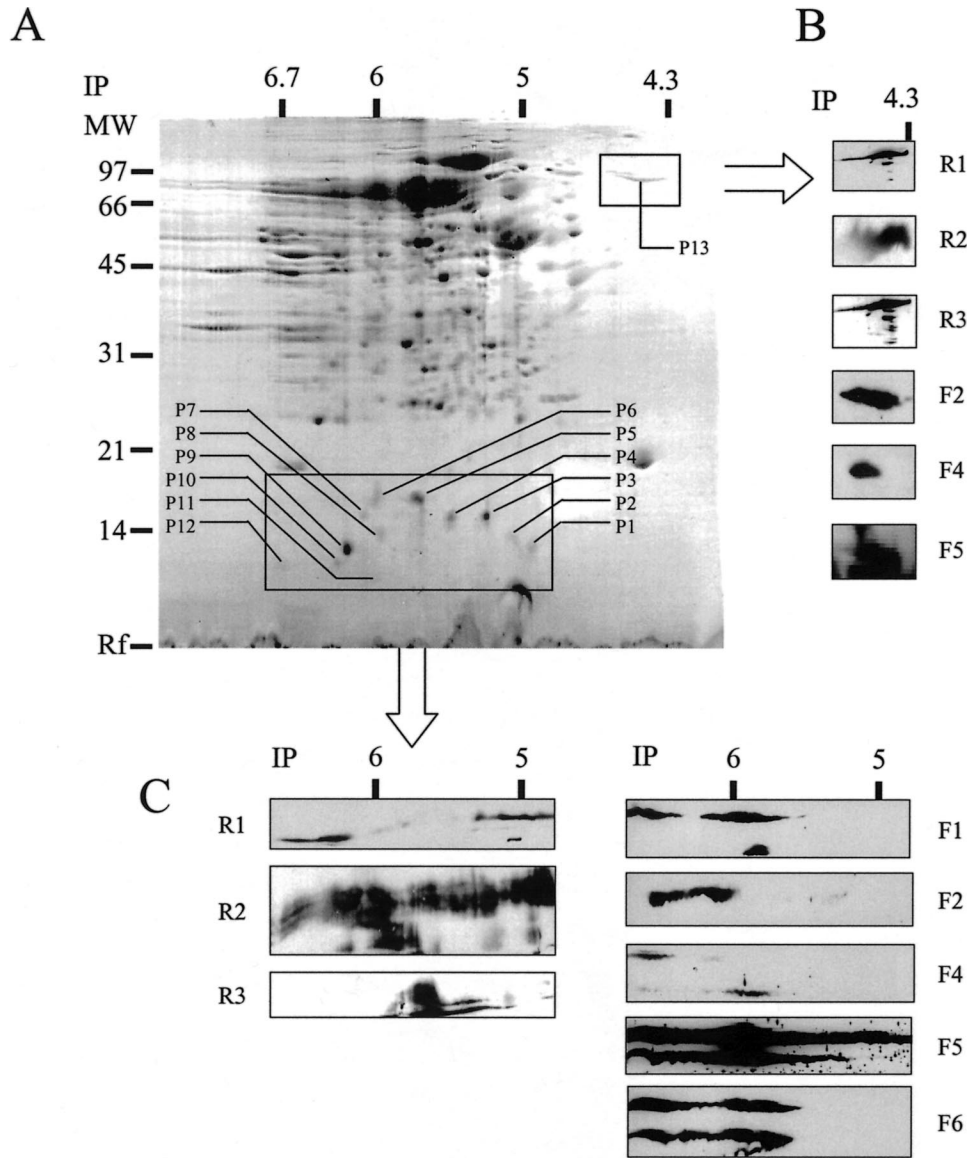


Figure 5. (A) Identification of fox sperm proteins P1 to P13 on two-dimensional electrophoresis gel stained with Coomassie blue. Molecular weight standards (kd) are indicated on the left, and the isoelectric point on the top of the gel. (B) Immunoblot of the box containing one 97-kd protein with rabbit sera R1, R2, and R3 (protocol 1), and fox sera F2, F4, and F5 (protocol 2). (C) Immunoblot of the box containing low-molecular weight proteins with rabbit sera R1, R2, and R3 (protocol 1), and fox sera F1, F2, F4, F5, and F6 (protocol 2). These 2 boxes contain highly antigenic proteins.

Table 1. Antigenicity of selected proteins as assessed by Western blotting with sera of rabbits and foxes on gel-purified proteins

Animals		Proteins												
		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13
Experiment 1 Rabbits	R1	-	-	NT	-	NT	-	-	++	-	NT	-	-	+++
	R2	-	-	-	-	+	+	-	-	-	-	-	-	+++
	R3	-	-	-	+	-	-	-	+++	-	-	+	-	+++
Experiment 2 Foxes	F1	-	-	-	+	-	-	+	+++	-	NT	+	NT	NT
	F2	-	-	NT	++	NT	-	-	++	-	NT	++	NT	+++
	F3	-	-	-	-	-	-	NT	-	-	-	-	-	-
	F4	-	-	-	-	-	-	-	+	-	NT	+	NT	+++
	F5	-	-	+	+++	+	-	-	+++	-	-	+++	-	+++
	F6	-	-	+	++	+	-	-	+++	-	-	-	-	NT

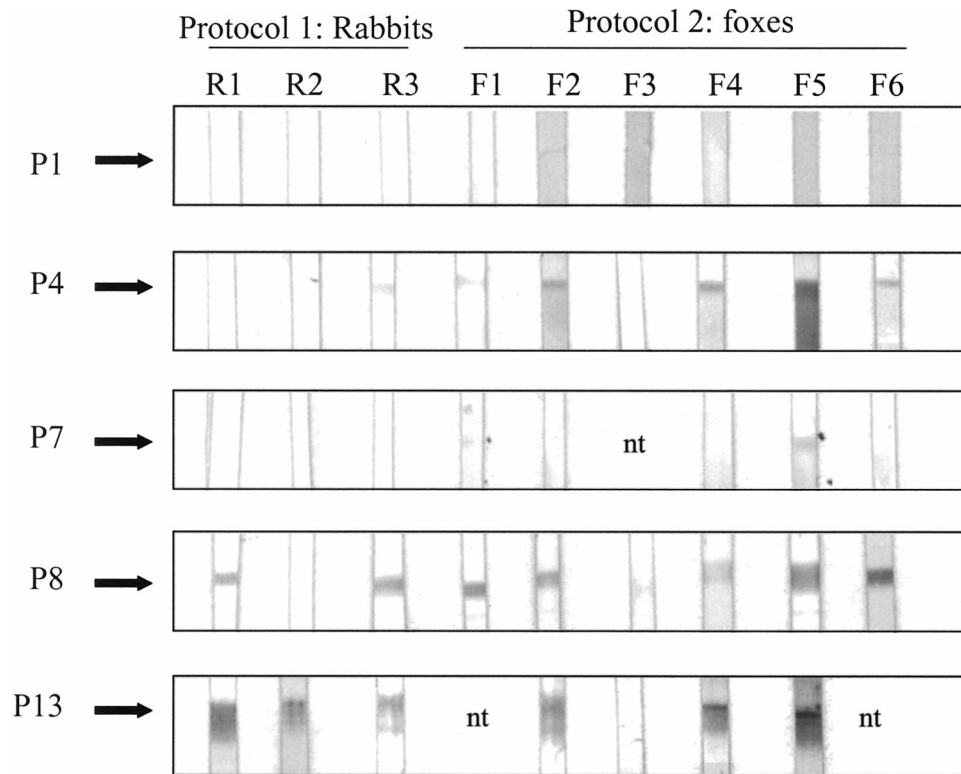


Figure 6. Antigenicity of the proteins P1, P4, P7, P8, and P13 assessed by Western blotting with rabbit sera R1 to R3 (protocol 1), and fox sera F1 to F6 (protocol 2) on gel-purified proteins (nt indicates not tested). Some proteins were not recognized by any sera (P1), others were recognized by some sera (P7), and still others were recognized by a large number of the sera from the 2 experiments (P4, P8, and P13).

number of recognized proteins increases after three booster injections and reaches a maximum on days 75 to 120. This result is consistent with those obtained by enzyme-linked immunosorbent assay for antigamete IgG of fox sera (Bradley, 1994). Another characteristic of the foxes' humoral responses is heterogeneity: only 8 out of the 25 bands stained by Western blot were recognized by 3 (or more) out of the 6 foxes. However, this result is due in part to the origin of the foxes, which are not conventional laboratory animals, and are as such, genetically more heterogeneous than the New Zealand line of rabbits. Heterogeneity of humoral response was also observed after immunization of elk with gamete antigens (Garrott et al, 1998) and seems to be characteristic of other wild species such as the fox.

Table 2. Properties of fox sperm proteins

Protein	Molecular Weight	IP
P3	16.8	5.3
P4	16.4	5.5
P5	16.9	5.8
P7	16.4	6.2
P8	14.7	6.0
P11	11.4	6.0
P13	97.0	4.3–4.6

The aim of this report was to identify highly antigenic proteins on fox sperm rather than to characterize the immune response. Primarily for this reason, we used sera only from immunized animals, and not from sham-immunized animals, so we used preimmune sera as a negative control for each immunized fox. Although a comparison between these sera could be interesting, we were limited by the small number of foxes available. Indeed, foxes are expensive animals and are relatively difficult to house. Similarly, for comparing humoral response among individual animals, we could rely only on the number of antigenic bands recognized by different antisera. However, the same band may represent different antibody isotypes, different epitopes, and indeed, different proteins of the same molecular weight. Nevertheless, it could be noted that, concerning the number of antigens recognized, there was no significant difference between males and females in the experimental conditions used.

Despite the heterogeneity of fox humoral responses, there exists a set of spermatozoa surface proteins that are recognized by a large proportion of fox sera. These highly antigenic proteins could probably be specific to mature sperm (Tung, 1998). In the second part of this study, the properties of these proteins were studied. For their characterization, two-dimensional gel electrophoresis tech-

niques were used. The additional resolution provided by these analyses showed more precisely the differences in the patterns of antigens recognized. By Western blotting of proteins purified by using two-dimensional electrophoresis and Coomassie blue staining of gels, 7 antigenic proteins (P3, P4, P5, P7, P8, P11, and P13) were identified by their molecular weights and isoelectric points.

This work applied an original approach for the selection of fox sperm antigens for use in a contraceptive vaccine. The fox sperm antigens described by others were previously identified in two ways: 1) by screening a fox testis cDNA library with a known DNA sequence (LDH-C4, PH-20), characterized in other species (Bradley et al, 1996, ten Have et al, 1998); and 2) by raising monoclonal antibodies to fox sperm (FSA-1, Beaton et al, 1994). Our approach was to use polyclonal antibodies, produced in rabbits and foxes, to select new highly antigenic fox sperm proteins. This allowed us to select 7 antigens that are relatively abundant (Coomassie blue staining) and highly antigenic for both male and female foxes.

In order to achieve a better characterization of these fox antigenic proteins, and before understanding their role in the fox-gamete interaction or considering their use in a contraceptive vaccine, their molecular structure, specificity, and function need to be investigated.

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