MOLECULAR CHARACTERIZATION AND IMMUNOLOCALIZATION OF SPERM SURFACE PROTEINS (SPAM-1 AND LDH-C_4) IN DOG

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ABSTRACT

Search for immunogenic contraceptives has demonstrated that two sperm surface proteins, *viz*., sperm adhesion molecule-1 (SPAM-1 / PH-20) and lactate dehydrogenase-C_4 (LDH-C_4) possessed immunocontraceptive properties. The molecular configuration and expression of these two proteins have been amply studied in humans, non-human primates, and laboratory animals, but least studied in dogs. The present study conducted on ejaculated semen depicts molecular characterization of SPAM-1 and LDH-C_4, and their expression in epididymis tissue and epididymis fluid in dog. Anti SPAM-1 raised in rabbit against human sperm SPAM-1 and anti LDH-C_4 raised in rabbit against a 54 amino acid long synthetic peptide sequence of LDH-C_4 were reacted with sodium dodycyl sulphate (SDS) and lithium di-iodosalicyalate (LDIS) sperm extracts (SESDS-SE & LDIS-SE), epididymal tissue extracts (ETE), and epididymal luminal fluid proteins (EFP) on immunoblots to characterize SPAM-1 and LDH-C_4. Anti SPAM-1 and anti LDH-C_4 reacted strongly with 46 kDa and 32 kDa bands both in SDS-SE and LDIS-SE. Anti LDH-C_4 identified a strong band of 36 kDa, both in SDS-SE and LDIS-SE, two weak bands of 30 kDa and 28 kDa in SDS-SE, and one weak band of 30 kDa in LDIS-SE. Anti SPAM-1 cross reacted to proteins of 46 kDa of EFP and to 32 kDa of ETE. LDH-C_4 antibody strongly cross reacted to protein of 36 kDa, and weakly to 46 kDa, 32 kDa, and 30 kDa of EFP. It also cross reacted strongly to 36 kDa, and weakly to 14 kDa proteins of ETE. The cellular distribution of SPAM-1 and LDH-C_4 in sperm plasma membrane, determined by indirect immunofluorescence on whole sperm, indicated localization of SPAM-1 mainly on head surface, and LDH-C_4 mainly in the post-acrosomal region and mid-piece of spermatozoa. Our study provided evidence on the existence of two sub units of SPAM-1 (46 kDa, 32 kDa) and three sub units of LDH-C_4 (36 kDa, 30 kDa, 28 kDa) in spermatozoa, and in the tissues and luminal fluid of all the three segments, *viz*., caput, corpus, and cauda of epididymis in dog.

KEY WORDS

Dog, Epididymis, Immunocontraception, LDH-C_4, SPAM-1,

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INTRODUCTION

Search for immunogenic contraceptives has demonstrated that two sperm surface proteins, *viz*., sperm adhesion molecule-1 (SPAM-1/ PH-20) and lactate dehydrogenase-C_4 (LDH-C_4) possessed immunocontraceptive properties (Primakoff *et al*., 1988, 1997).

PH-20 is a sperm surface protein, linked to glycosyl phosphatidylinositol (Lin *et al*., 1994), recognized as a bifunctional protein with hyaluronidase activity, and its role is related to cumulus penetration and zona binding (Meyer *et al*., 1997). In sperm metabolism, lactate dehydrogenase C_4 (LDH-C_4) is a key enzyme, distributed specifically in testis and is highly immunogenic. Sperm-specific lactate dehydrogenase or lactate dehydrogenase C_4 (LDH-C_4) exists in testis and spermatozoa of mammalian and avian species, the function of which relates to energy metabolism and sperm capacitation (Yong *et al*., 2008).

There have been ample studies on SPAM-1 (Primakoff *et al*., 1985; Overstreet *et al*., 1995; Hunnicutt *et al*., 1996; Meyer *et al*., 1997; Sabeur *et al*., 1997; Evans *et al*., 2003) and LDH-C_4 (Sawane *et al*., 2002; Yong *et al*., 2008) in many mammalian species, but least studied in dogs. Studies on the molecular and biochemical characteristics of these proteins are crucial, before SPAM-1 and LDH-C_4 could be recommended as potential contraceptive agents in dog. The aim of the present work was to determine SPAM-1 and LDH-C_4 in dog sperm using polyclonal antibodies against a human SPAM-1 immunogen and a 54 amino acid long synthetic peptide of LDH-C_4, and their interaction with epididymal tissue and epididymal fluid.

MATERIALS AND METHODS

Collection and processing of semen: Semen of four healthy adult mongrel dogs weighing about 16-20 Kg, aged 2-3 years, was collected for this study. The dogs were housed in concrete floored kennels with access to outside runs, fed on commercial dog feed (Nutripet), and had free access to drinking water, for 4 weeks prior to semen collection, for acclimatization. The semen (entire 2nd sperm rich fraction and part of 3rd fraction) was collected by manual manipulation in clean graduated tube attached to a glass funnel. Five ejaculates were collected from each dog with a minimum interval of 4 days. Semen was immediately centrifuged to separate out spermatozoa and seminal plasma for the analysis.

Preparation of sperm extracts: The ejaculated spermatozoa (500 x10^6), washed twice with PBS (pH 7.4) were suspended in

1.0 ml of 2% SDS in 62.5 mM Tris-HCl (pH 8.0), containing protease inhibitors (1mM PMSF, 25 mM benzidine and 10mM aprotinin, and 10mM pepstatin + 5mM soyabean trypsin inhibitor), sonicated at 4°C (20 W, thrice for 20 seconds each) and centrifuged at 16,000 g for 30 minutes to prepare sodium dodecyl sulphate sperm extract (SDS -SE).

For preparation of lithium di-iodosalicylate sperm extract (LDIS-SE), ejaculated spermatozoa (500 x10^6), washed twice with PBS (pH 7.4) were suspended in 1.0 ml of 0.3 M LDIS in 50 mM Tris-HCl (pH 8.0) containing protease inhibitors (1mM PMSF, 25 mM benzidine and 10mM aprotinin, 10mM pepstatin + 5mM soyabean trypsin inhibitor), agitated at room temperature (25±3 °C) for 30 minutes, and then at 4°C for two hours. The sperm suspension was centrifuged at 16,000 g for 30 minutes and the supernatant was dialyzed against 0.15M lithium chloride and 50 mM Tris-HCl (pH 8.0) containing 0.15M NaCl and 1 mM EDTA.

The sperm extracts (SDS-SE) and (LDIS-SE) were stored in 0.1 ml fractions at -20 °C till further use.

Extraction of epididymal tissue proteins and purification of epididymal fluid proteins: Gonads of one of the dogs, which were not donating semen, were removed surgically, and epididymii removed from the testis were divided in to three parts, i.e. caput, corpus and cauda. All the blood vessels of three regions of epididymis were removed and thoroughly washed with phosphate buffered saline (PBS) at pH 7.4.

The tissues were incised, and the fluid containing spermatozoa was collected in vials with 1.0 ml PBS. The spermatozoa were separated from epididymal fluid by centrifuging at 10,000 g for 30 minutes. Proteins were separated from epididymal fluid through precipitation by saturated ammonium sulphate $(NH_4)_2SO_4$. The precipitated proteins were suspended in known volume of PBS.

The previously washed epididymal tissue was dried between folds of filter paper. One gram of dried tissue was sonicated (20 watts, 2 minutes) at 4°C in 0.02M Tris-HCl buffer (pH 7.5), 0.5 mM dithiothretol, 0.2 mM EDTA, 2% SDS and protease inhibitors (1mM PMSF, 25 mM benzidine and 10mM aprotinin, 10mM pepstatin+5mM soyabean trypsin inhibitor). The sonicated samples were centrifuged at 16,000 g for 30 minutes and tissue extracts were stored at -20 °C till further use.

SDS-PAGE and Immunoblotting: Anti SPAM-1 (raised in rabbit against human sperm SPAM-1) and Anti LDH-C_4 (raised in rabbit against synthetic peptide) were

reacted with sperm extracts, epididymal fluid, and epididymal tissue extracts (Laemmli, 1970; Towbin *et al*., 1979). Proteins separated by SDS-PAGE under reducing conditions were transferred to nitrocellulose / PVDF membrane using wet electrophoresis transfer apparatus (Clever Scientific Co, UK, VS10WD) at 100 V for 2.5 hours. Transfer quality was checked by 0.2% ponceau dye and proteins were blocked in 3% BSA as blocking solution for overnight at 4°C.

The membrane was washed with PBS + 0.05% Tween-20, and was incubated in 1: 1000 diluted primary antibodies (Anti SPAM-1 raised in rabbit against human sperm SPAM-1 and anti-lactate dehydrogenase raised in rabbit against synthetic peptide sequence (Given below in parenthesis), purchased from Sigma-Aldrich, Saint Louis, USA) for 2.5 hours. The membrane was again washed thrice with PBS + 0.05% Tween-20, and incubated with 1:10000 anti-rabbit IgG as secondary antibody for 45 minutes. It was washed thrice with PBS + 0.05% Tween-20 and incubated with substrate (0.05% Diaminobenzidine + 0.015% 4-Chloro Napthol + 0.06% Hydrogen Peroxide) for 10 minutes. Gel images were captured on gel doc (Syngene International Ltd, UK, SYDR4) using Gene Snap image acquisition software and analyzed by using GeneTools gel analysis software.

Peptide sequence: (TYIVWKISGLPVTRVI GSGCNLDSARFRYLIGEKLGVHPT SCHTSC HGWIIGEH)

Immunolocalization of antigenic proteins: Immunolocalization of antigenic proteins was done using FITC labeling (Verdier *et al*., 2002). Smears of washed dog spermatozoa were prepared on glass slides, air-dried, and fixed in ethanol for 30 minutes. Slides were then covered with PBS containing 1% BSA for 45 minutes to block nonspecific antibody binding. They were then incubated at room temperature (25±3 °C) in a humidified chamber for 2 hours with Anti SPAM-1 and Anti LDH-C_4 diluted to 1:1000. 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 a Leica fluorescent microscope (Leica, Germany) and images were captured on Leica digital camera. Negative controls with either primary or secondary antibody were also run.

RESULTS

Immunoblotting of anti SPAM-1 and anti LDH-C_4 with sperm extracts: Non–reducing SDS-PAGE of SDS-SE and LDIS-

SE indicated that the molecular weights of sperm proteins in dogs were of 173, 116, 86, 72, 62, 60, 55, 46, 42, 36, 32, 28, 23, 20, and 17 kDa (Figure-1). Anti SPAM-1 cross reacted strongly to two protein bands of 46 kDa and 32 kDa both in SDS-SE and LDIS-SE under reducing conditions (Figure-2), while anti LDH-C_4 cross reacted strongly to a protein band of 36 kDa both in SDS-SE and LDIS-SE, and weakly to two protein bands of 30 kDa and 28 kDa in SDS-SE and to one band of 30 kDa in LDIS-SE under reducing conditions (Figure-3). This indicated that there were two sub units of SPAM-1 and three sub units of LDH-C_4 in dog sperm.

Immunoblotting of anti SPAM-1 and anti LDH-C_4 with epididymal fluid and tissue proteins: Anti SPAM-1 cross reacted to 46 kDa protein band in the luminal fluid of caput, corpus, and cauda, and to 32 kDa band of SDS extracts of caput, corpus, and cauda tissues of epididymis (Figure-2). Anti LDH-C_4 strongly cross reacted to 36 kDa protein band and weakly to 46 kDa, 32 kDa, and 30 kDa protein bands in the luminal fluid of caput, corpus, and cauda of epididymis. It strongly cross reacted to 36 kDa protein band and weakly to 14 kDa protein band of SDS extracts of caput, corpus, and cauda tissues of epididymis (Figure- 4).

Immunolocalization of SPAM-1 and LDH-C_4 in dog spermatozoa: The cross reaction of anti SPAM-1 with dog spermatozoa on immunofluorescence slides gave very strong signals on the entire head surface, and a signal of very weak intensity on the tail (Figure- 5). This indicated localization of SPAM-1 mainly on the head surface of the sperm in dog. A very strong signal was obtained on post-acrosomal region and mid piece with anti LDH-C_4 (Figure-6), indicating localization of LDH-C_4 mainly in the post-acrosomal region and mid-piece. A signal of weak intensity was also detected on the anterior of head and tail. No fluorescence was observed in the negative or positive controls.

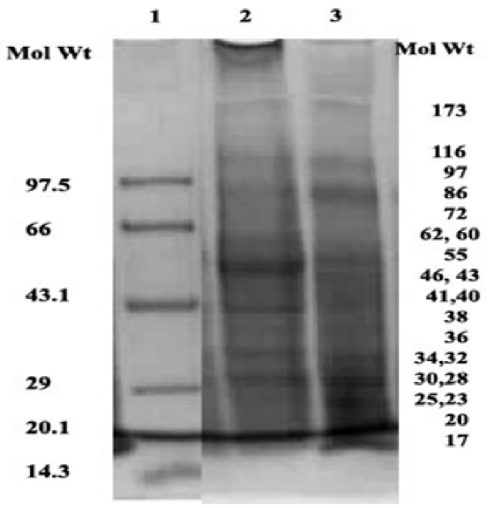

Figure-1. SDS-PAGE of sperm extracts of dog. Lane-1: Standard, Lane 2: LDIS-SE, Lane-3: SDS-SE.

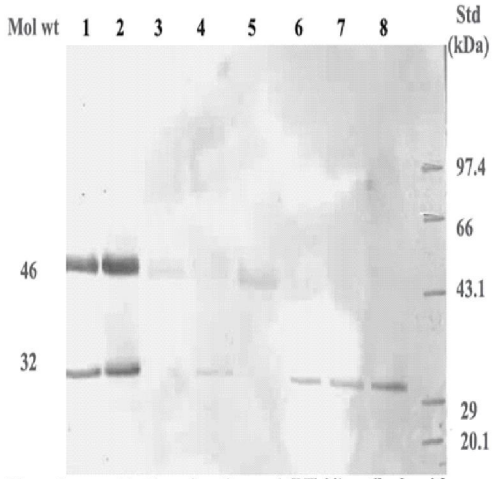

Figure-2. Immunoblotting of anti SPAM-1 (PH-20) antibody with sperm membrane extracts (SME), epididymal tissue extracts (ETE), and epididymal fluid (EF). Lane-1: SDS-SME, Lane-2: LDIS-SME, Lane-3: Cauda EF, Lane-4: Corpus EF, Lane-5: Caput EF, Lane-6: Cauda ETE, Lane-7: Corpus ETE, Lane-8: Caput ETE .

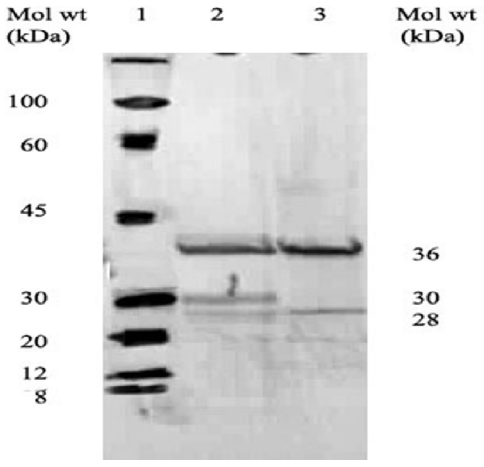

Figure-3. Immunoblotting of anti-LDHC with SDS-SME and LDIS-SME. Lane-1: Standard, Lane-2: SDS-SME, Lane-3: LDIS-SME.

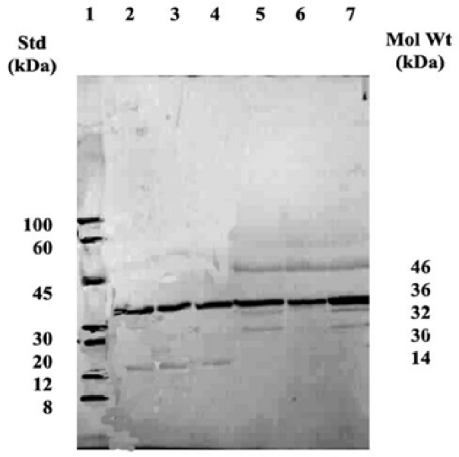

Figure-4. Immunoblotting of anti-LDHC with epididymal tissue extracts (ETE) and epididymal fluid (EF). Lane-1: Standard, Lane-2: Caput ETE, Lane-3: Corpus ETE , Lane-4: Cauda ETE, Lane-5: Caput EF, Lane-6: Corpus EF, Lane-7: Cauda EF.

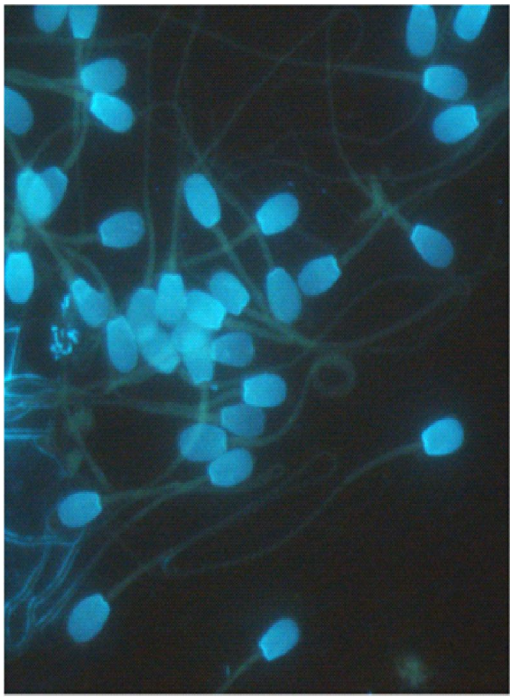

Figure-5. Immunolocalization of SPAM-1, detected by anti SPAM-1.

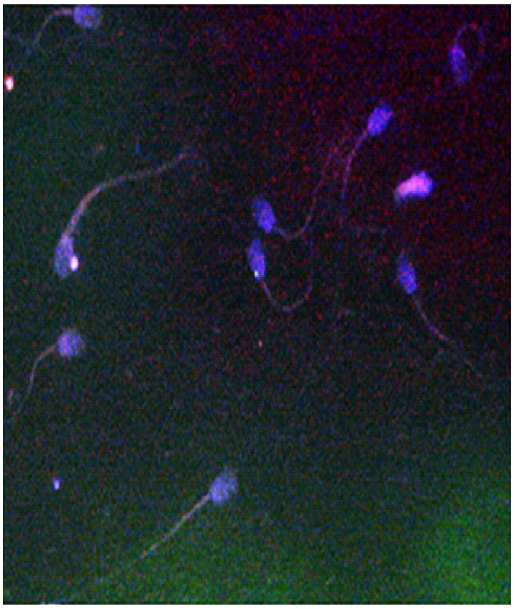

Figure-6. Immunolocalization of LDH-C_4, detected by anti-lactate dehydrogenase.

DISCUSSION

Immunoblotting of anti SPAM-1 and anti LDH-C_4 with sperm extracts: Initially characterized by Primakoff *et al*. (1985) in guinea pig sperm, SPAM-1 has been found in many other species, including cynomolgus macaques, humans and canines (Overstreet *et al*., 1995; Sabeur *et al*., 1997, 2002). Purified PH-20 of guinea pig sperm had been found to exist in three forms, separable on SDS-PAGE, i.e., a major form of 64 kDa, a minor form of 56 kDa, and fragments of 41-48 kDa, and 27 kDa.

In our study, reaction of anti SPAM-1 with 46 kDa and 32 kDa proteins of SDS-SE/ LDIS-SE indicated that 46 kDa sub unit of dog sperm's SPAM-1 is a major form and 32 kDa is a minor form. It was not in agreement with the findings of Sabeur *et al*. (2002), who found a major band of 50 kDa, and three bands of 42, 124, and >209 kDa of PH-20 in dog sperm. This difference could be due to the type of antibody (antisera raised in rabbits against recombinant cynomolgus macaque) used in his experiment.

Lalancette *et al.* (2001) characterized PH-20 of bull spermatozoa as 80 kDa protein, and Guillaume *et al.* (2005) further consolidated it with his theory that some unidentified modifications of this protein occurring in the epididymis could result in the decrease of the mass of PH-20. Differences also existed between the dog sperm SPAM-1 and SPAM-1 of other species, because dog sperm protein migrates at 46 and 32 kDa on SDS-PAGE, whereas SPAM-1 in most other species migrates at 58–68 kDa (Overstreet *et al*., 1995).

Three sub units of LDH-C_4 (36 kDa, 30 kDa, 28 kDa) were detected in dog sperm in our study. In rat, LDH-C_4 has been found to exist in two forms of isoenzymes, *viz*., C4 and A1C3 (Yong *et al*., 2008). Therefore, 36 kDa, 30 kDa, and 28 kDa proteins detected by anti LDH-C_4 in ejaculated dog spermatozoa in our study could possibly be the three isozymes of LDH-C_4.

In our previous study (Cheema *et al.,* 2012), 46 kDa and 32 kDa (PH-20 sub units), and

36 kDa, 30 kDa, and 28 kDa (LDH-C_4 sub units) were also characterized as antigenic proteins with iso-antiserum.

Immunoblotting of anti SPAM-1 and anti LDH-C_4 with epididymal fluid and epididymal tissue proteins: SPAM-1 was earlier believed to be testis-specific. Later, it was reported that it was also synthesized in the epididymal epithelium and released in the luminal fluid (Deng *et al*., 2000). Epididymal SPAM-1 (ES), like testicular SPAM-1 (TS), was shown to have hyaluronidase activity at neutral pH.

Epididymal proteins are involved in the morphological, physiological, and biochemical changes that mammalian sperm undergo during epididymal transit, where they acquire motility and fertilizing capability (Kirchhoff *et al*., 1998). It is thus possible that ES might have a role in sperm maturation. This might occur if ES binds to sperm either as a unique isoform or for the purpose of enhancing SPAM-1 of testicular origin. In view of above observations, it can be inferred that 32 kDa tissue protein of dog is synthesized by epididymal epithelium and binds to sperm during transit, whereas 46 kDa luminal fluid proteins is TS, and is released into luminal fluid from the sperm membrane.

The cross reaction of anti Spam-1 with 32 kDa protein of caput-, corpus- and cauda epididymis further indicated that PH-20 sub unit in dog is synthesized at three epididymal regions, and supported the observations of Evans *et al*. (2003). Deng *et al*. (2000) also supported the possibility that there might be two sources of SPAM-1 in the luminal fluid: molecules that come from sperm and those that are released from the epididymis. Zhang and Martin-DeLeon (2003) reported that approximately 40% of SPAM-1 in the epididymal luminal fluid is soluble and is of the same molecular weight as the insoluble SPAM-1 which has an intact GPI anchor.

Earlier studies on LDH-C_4 revealed its presence in testis and in spermatozoa, but not in other tissues or cells (Yong *et al*., 2008). Our studies indicated cross reaction of anti LDH-C_4 with 46 kDa / 36 kDa / 32 kDa / 30 kDa protein bands of caput-, corpus-, and cauda- luminal fluids, and 36 kDa / 14 kDa protein bands of SDS extracts of caput-, corpus- and cauda- tissues. This showed that testicular 36 kDa sub unit of LDH-C_4 in dog is being transported in epididymal fluid, which is later absorbed by the epididymal epithelium.

The reaction of anti LDH-C_4 with 30 / 32 kDa sub units of epididymal fluid indicated their release into the lumen but with some alteration in the molecular structure. Cross reaction of anti LDH-C_4 with 46 kDa protein of luminal fluid and 14 kDa of tissue in dog indicated homology of LDH-C_4 with some other proteins like LDHA / LDHB, as

structural studies of LDH-C_4 had confirmed that it was homologous to the LDHA and LDHB sub units, having 72.5% identity with LDHA and 64.5% identity with LDHB in mice, and 75.3% identity with LDHA, and 69.8% identity with LDHB in humans (Pan *et al*., 1983).

Immunolocalization of SPAM-1 and LDH-C_4 in dog spermatozoa: The cross reaction of anti SPAM-1 with dog spermatozoa indicated its localization mainly on the entire head surface in dog. It is similar to the fluorescence pattern reported earlier in mouse, human, macaque, and canine (Lin *et al*., 1994; Overstreet *et al*., 1995; Sabeur *et al*., 2002).

On the other hand, localization of SPAM-1 has been reported at the post acrosomal region in guinea pig (Myles and Primakoff, 1984) and in stallion (Meyers and Rosenberger, 1999). But indirect immunofluorescence on non-permeabilized spermatozoa revealed the localization of PH-20 at the post acrosomal region of the head in bull and on the anterior region of the head of spermatozoa fixed/ permeabilized with ethanol (Lalancette *et al*., 2001). Lin *et al*. (1994) corroborated that the presence of PH-20 on the anterior head of acrosome-intact sperm plays a potential role in penetration of the extracellular matrix (ECM) surrounding the oocyte, as only acrosome-intact cells can pass through the cumulus matrix.

The cross reaction of anti LDH-C_4 with dog spermatozoa indicated localization of LDH-C_4 mainly in the post-acrosomal region and mid-piece. Several studies have also provided evidence that LDH-C_4 might be localized in the matrix of sperm type mitochondria (Burgos *et al*., 1995). Immunohistofluorescence studies with antibodies produced with peptides designated MC5-15 and MC211-220 detected strong signals in the principal piece of the spermatozoa (Odet *et al*., 2008) and weak signal in the midpiece. In the past, PH-20 and LDH-C_4 have shown promising result as an immunocontraceptive agent (Primakoff *et al*., 1988; 1997; Goldberg, 1990; O'Hern *et al*., 1995).

CONCLUSION

Our results provide evidence regarding the presence of two sub units of SPAM-1 (PH-20) and three sub units of LDH-C_4 in dog's sperm and their interaction with epididymal tissue and epididymal luminal fluid during transit through epididymis indicating their role in fertility of dog sperm.

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