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In-silico Characterization and Epitope Profiling of Differentially Expressed Plasma Membrane Proteins of 'X' and 'Y' Chromosome Bearing Bovine Sperm

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ABSTRACT

Background: For the development of an immunological sperm sorting technology detection of a target protein in either X or Y chromosome-bearing sperm is mandatory. Detailed study of such proteins and finding antibodies against those sex-specific and differentially expressed proteins is the most essential step to looking forward to the immunological sexing of sperm.

Methods: The specific and differentially expressed plasma membrane proteins of bovine sex-sorted semen were selected after reviewing previous research publications related to the proteomic profile of bovine-sexed semen. The *in-silico* characteristics such as physicochemical properties, primary, secondary, tertiary structures, membrane topology, sub-cellular localisation, domain analysis, protein interactions and epitope profiling, both linear and discontinuous peptides were done for selected plasma membrane proteins.

Result: The results showed the localisation of proteins, their structure, polarity, stability, domains, function and probable epitopes of various selected X and Y bovine sperm plasma membrane-specific and differentially expressed proteins. For X-specific protein CLRN3, 6 and 4 linear and discontinuous epitopes and for Y chromosome-specific protein, SCAMP1, 6 and 8 linear and discontinuous epitopes were predicted, respectively. The results of *in-silico* characterization and epitope prediction of various selected X and Y bovine sperm plasma membrane-specific and differentially expressed proteins would be helpful in the selection of some efficient antigenic proteins to be used for further X and Y sperm-specific antibody production or synthesis of antibodies artificially.

Key words: Differential proteins, Epitope profiling, *In silico* characterization.

INTRODUCTION

Now with the automation of both agriculture and transport, the oxen or bulls are not required for the draught work and therefore only female calves are being kept and reared by farmers for milk production. Beef is normally produced from male calves of buffalo or cattle so the farmers generally sell them at low prices or sometimes dispense them as strays. This gives rise to an animal welfare issue as well as ethical uneasiness for animal lovers. Various religious factions also forbid the slaughter of bulls, leading to an increase in communal tensions. It is also quite clear that global agriculture and the associated animal industries will face a crunch in the next few decades. As population pressures mount, the competition for land by both man and animals will intensify (Vishwanath and Moreno, 2018). So, expanding the livestock industry without putting too much pressure on the agricultural land is essential. Controlling the sex of the offspring to influence the birth rate of the desired gender is one of the likely ways to do so sharma *et al.* (2018) and it will allow greater production efficiency and flexibility in herd management (Carvalho *et al.,* 2014, Naidu *et al.,* 2021). Thus, improvement in semen sexing technology will economically assist cattle farmers and help avoid risks to communal harmony (Rai, 2018; Yekti *et al.,* 2022; Sharma *et al.*, 2022).

One of the major limitations of current semen sexing technology is its slow speed of the process concerning the number of viable sperm required for artificial insemination (Sharma and Sharma, 2016; Kumar *et al.,* 2016; Sharma *et al.,* 2024). In a given amount of time, only some sperm can be evaluated accurately due to the physical limitations, as each sperm has to pass through a nozzle to be analysed

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by the detecting laser (Garner *et al.,* 2012). Another limitation of sex-sorted semen is a reduction in the fertility of semen, as there are at least an additional 20 different sub-processes involved in the sex-sorting procedure which includes an extended holding time before staining, exposure to a laser beam to induce fluorescence, separation into X and Y-sperm and exposure to an electrical field for making a relatively pure population into an appropriate vessel (Vishwanath and Moreno, 2018; Seidel and Garner, 2002). Due to these shortcomings (Bhat and Sharma, 2020; Rawat and Sharma 2020; Chaudhay *et al*., 2023; Rawat *et al.*, 2023; Chaudhay *et al*., 2023), it is very meaningful to establish an efficient, inexpensive, convenient and less invasive approach for sorting sperm (Blecher *et al.,* 1999; Yang *et al.,* 2014). Since current semen sexing techniques efficiently sort X and Y-bearing sperm populations, therefore, it also provides us with an opportunity to discover differences in proteins between separated populations of X and Y-spermatozoa, which can be used in designing immunological methods to sort sperm cells, which can prove very fruitful for the future of sperm sorting (Sharma and Sharma, 2024).

The presence of different proteins in the X and Y sperm populations forms the basis of the immunological approach to sperm sexing (Seidel and Johnson, 1999; Sharma and Hadiya, 2024). If we are to isolate or identify different proteins between X and Y sperm, these will serve as a marker, against which antibodies could be developed. This is the basic principle behind the immunological method of sperm sexing. The accessibility of antibodies to the selected protein targets is the determining factor in the possibility of detection and separation of a known cell by using specific antibodies (De Canio *et al.,* 2014; Yadav *et al.,* 2017). The specific surface components of separated sperm will play an important role in this aspect. Indirect shreds of evidence have implied that differences in surface proteins might exist between X and Y-sperm (Grant *et al.,* 2008; Robbins *et al.,* 2008; Zhang *et al.,* 2008) based on these if a set of antibodies can recognise the cellular proteins, then a more efficient immunological approach may be developed as a substitute and perhaps more effective way for searching SSP and consequently developing a better method of sperm sexing (Beerli *et al.,* 2008).

For the development of an immunological sperm sorting technology detection of a target protein in either X or Y chromosome-bearing sperm is mandatory. There is uncertainty in the direct evidence of sex-specific proteins, but various methods could be applied to searching for sex-specific antigens that are expressed at low levels (Sang *et al.,* 2010). Detailed study of such proteins and finding antibodies against those sex-specific and differentially expressed proteins is the most essential step to looking forward to the immunological sexing of sperm. One such approach is *in silico* study of the differentially expressed proteins of sexed semen. In biology and other experimental studies, an *in-silico* experiment is performed on a computer or by using computer simulation. By using different software and tools one can successfully predict the location, domains, interactions, structures, epitopes and binding of various molecules or antibodies to the protein being studied. By assessing the differentially expressed proteins *in silico,* one can even predict the most suitable protein against which the antibodies should be synthesized to use them for developing an effective method of sperm separation. An *in silico* study can prove to be an essential tool to guide the research for the development of an immunological method of sperm sexing. There is no doubt that the identification of protein differences, especially a difference between plasma membrane proteins of X and Y-bearing spermatozoa will pave the way for the development of a better and more promising immune sexing technique for spermatozoa.

MATERIALS AND METHODS

In-silico analysis of differentially expressed proteins on X and Y bovine sperm

Twenty-eight plasma membrane proteins, which were differentially expressed in X or Y chromosome-bearing bovine sperm were selected for this study. Out of these twenty-eight proteins, two proteins, CLRN3 (Clarin3) and SCAMP1 (Secretory Carrier Membrane Protein 1) were found to be specific for X and Y chromosome-bearing bovine sperm, respectively and twenty-six were up-regulated in either X or Y chromosome bearing bovine sperm.

Retrieval of the protein sequences

The sequences of the proteins were retrieved from NCBI (National Centre for Biotechnology Information) and UniProt databank (https://www.uniprot.org/) in FASTA format (http://www.ncbi.nlm.gov/). The BLAST programme was run using the NR database of NCBI to identify homologous proteins and it was found that ATP-binding cassette sub-family. A member 3-like had 100% query coverage and 96.07 per cent identity to this protein.

Physicochemical properties of the proteins

The proteins' primary structure and physicochemical properties were determined using the AA-Prop server (http://www.biogem.org/tool/aa-prop/).

Determination of the secondary and tertiary (three-dimensional) structures of the protein

To predict the secondary and tertiary structure of proteins CFSSP and RaptorX servers were used respectively (http://raptorx.uchicago.edu/; http://www.biogem.org/tool/chou-fasman/). The tertiary structures of the proteins were first retrieved from PDB and the remaining were prepared using Raptor X server and Modeller software. The stereochemical properties of the modelled protein were verified using the Ramachandran plot.

Membrane protein topology, subcellular localization and domain prediction

The consensus prediction of membrane protein topology and signal peptides was done by using TOPCON'S server (Tsirigos *et al.,* 2015; https://topcons.cbr.su.se/pred/).

Prediction of the subcellular location of the proteins was done by the CELLO (subcellular localization predictor) tool (Yu *et al.,* 2004; 2006, http://cello.life.nctu.edu.tw). The domain characterisation of the proteins was done by using the SMART database (Letunic *et al.,* 2018; 2021, http://smart.embl-heidelberg.de).

Protein-protein interaction of the selected proteins

STRING database version 11 was used to find out the interactions between proteins, both direct as well as indirect (https://string-db.org/).

Epitope prediction of the proteins

Prediction of linear epitopes from protein sequence was done using IEDB, Bepipred linear epitope prediction version 2.0 and epitope prediction based upon structural protrusion was done by using the ElliPro tool.

RESULTS AND DISCUSSION

Various bioinformatics tools and software like AA Prop, CELLO, RaptorX, SMART, STRING, CFSSP, BepiPred, ElliPro and TOPCONS were used to conduct the study. For the study, different publications related to the bovine spermatozoa proteins were reviewed and 28 differentially expressed and sex-specific plasma membrane proteins of X or Y chromosome-bearing bovine sperm were selected (De Canio *et al.,* 2014; Chen *et al.,* 2012; Laxmivandana *et al.*, 2021; Quelhas *et al.,* 2021; Shen *et al.,* 2021).

Physicochemical properties of the proteins

The physicochemical results for all 28 proteins (Fig 1 and Table 1) showed that a total of 12 proteins have an instability index of above 40.

The secondary structure of the proteins

Secondary structures of all the proteins were predicted by the CFSSP server and RaptorX server. The secondary structures (Fig 3) that were obtained show the linear peptide chain of protein with changing alpha helix, beta pleated sheet and turns and coils. It was observed that 19 proteins had more alpha helices than beta sheets. These proteins were A-kinase anchor protein 3, L-lactate dehydrogenase A, Calmodulin, Outer dense fibre protein 2, Triosephosphate isomerase, SPACA1, L-asparaginase, ATP synthase subunit beta, mitochondrial, F-actin-capping protein subunit beta, Transmembrane protein 190, Keratin, type I cytoskeletal 19, Desmoplakin, Elongation factor 1-alpha1, RAB2B, member RAS oncogene family, Voltage-dependent anion-selective channel protein 1, SCAMP1, Keratin, type II cytoskeletal 5, Carboxypeptidase and uncharacterised protein. Due to a large number of alpha helices, these proteins would be able to make a more stable Protein-Lipid complex than other proteins (Tempra *et al.*, 2021).

Tertiary structures of the proteins

The tertiary structures of the proteins were first retrieved from PDB and Swiss model data banks and the rest which were not available on this database were predicted by the RaptorX server and Modeller software. The tertiary structure of Calmodulin was retrieved from PDB with PDB id 1PRW while Tubulin beta 4B was retrieved from the Swiss Model data bank with identifier Q3MHM5. The three-dimensional structures of all other proteins were modelled using Raptor X except for Uncharacterised protein and Desmoplakin, which were modelled using Modeller software due to their large number of amino acids. The modelled structures were then verified using the Ramachandran plot and it was found that all the modelled structures have highly preferred observations above 90% (Anderson *et al.,* 2005). The tertiary structures of these proteins were further used for the prediction of discontinuous epitopes of the proteins.

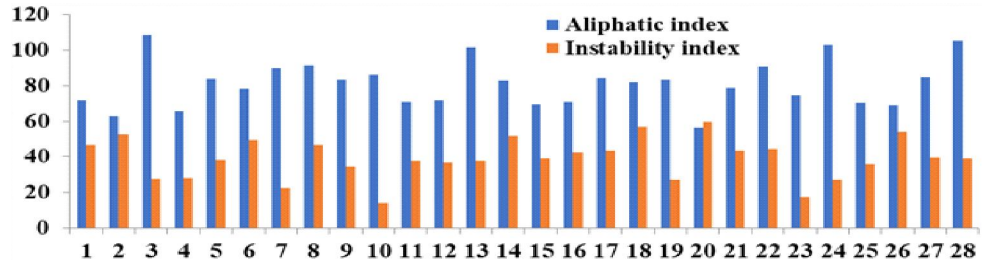
Membrane protein topology

The consensus predictions of membrane protein topology that is the locations and in/out the positioning of the membrane-spanning regions and signal peptides were done by using TOPCON'S server. It helps to obtain basic structural knowledge of the trans-membrane proteins. For the TM region of proteins, this method is of importance as there is difficulty in attaining structural information experimentally (Tsirigos *et al.,* 2015). It was observed that in proteins, Seminal plasma protein PDC 109, SPACA1, Transmembrane protein 190, Seminal plasma protein BSP-30 kDa, Leucine-rich repeat and fibronectin type III domain containing 2 and CLRN3 some sequence of amino acids function as signal peptides. Proteins like A-kinase anchor protein 3, Calmodulin, Glyceraldehyde 3 phosphate dehydrogenase testis-specific, Outer dense fibre protein 2, Triosephosphate isomerase, Tubulin alpha 3, L-asparaginase, Tubulin beta-4B chain, Tubulin beta 4ª, ATP synthase subunit beta, mitochondrial, F-actin-capping protein subunit beta, Tubulin beta 2B, Keratin, type I cytoskeletal 19, Desmoplakin, Elongation factor 1-alpha 1, RAB2B, member RAS oncogene family, Voltage-dependent anion-selective channel protein 1 and Keratin, type II cytoskeletal 5 have their membrane topologies as completely outside the membrane and can be of interest for the prediction of specific antibodies for sorting of bovine sperm.

Sub-cellular localization of the proteins

The function of a protein is often linked to its sub-cellular location but determining the sub-cellular location of a protein experimentally is a bit tedious. For this purpose, the CELLO tool was used (Yu *et al.,* 2004; 2006, http://cello.life.nctu.edu.tw).

It was observed that 5 proteins, *i.e*., A-kinase anchor protein 3, Outer dense fibre protein 2, F-actin-capping protein subunit beta, Desmoplakin and Keratin, type II cytoskeletal 5 were localised in nuclear region; 3 proteins, *i.e*., Seminal plasma protein PDC 109, Seminal plasma protein BSP-30 kDa and Transmembrane protein 190 were in extracellular region; 12 proteins, *i.e*., L-lactate dehydrogenase A, Calmodulin, Glyceraldehyde 3 phosphate dehydrogenase testis-specific, Triosephosphate isomerase, Tubulin alpha 3, L-asparaginase, Tubulin beta-4B chain, Tubulin beta 4ª,



1 represents A-kinase anchor protein 3, 2-Seminal plasma protein PDC 109, 3-L-lactate dehydrogenase A, 4-Calmodulin, 5-Glyceraldehyde 3 phosphate dehydrogenase testis-specific, 6-Outer dense fibre protein 2, 7-Triosephosphate isomerase, 8-SPACA1 Sperm acrosome membrane-associated protein1, 9-Tubulin alpha 3, 10-L-asparaginase, 11-Tubulin beta"4B chain, 12-Tubulin beta 4ª, 13-ATP synthase subunit beta, mitochondrial, 14-F-actin-capping protein subunit beta, 15-Tubulin beta 2B, 16-Transmembrane protein 190, 17-Keratin, type I cytoskeletal 19, 18-Desmoplakin, 19-Elongation factor 1-alpha 1, 20-Seminal plasma protein BSP-30 kDa, 21-RAB2B, member RAS oncogene family, 22-Leucine-rich repeat and fibronectin type III domain containing 2, 13-Voltage-dependent anion-selective channel protein 1, 24- CLRN3, 25- SCAMP1, 26- Keratin, type II cytoskeletal 5, 27- Carboxypeptidase and 28 represents Uncharacterised protein.

Fig 1: Predicted aliphatic index and instability index of the proteins.

Table 1: Physicochemical properties of the proteins.

Protein name	Mol. Wt.	pI	Negatively charged residue	Positively charged residues	Molecular formula	Aromaticity
A kinase anchor protein 3	94672.3	6.28	111	104	$C_{4128}H_{6575}N_{1149}O_{1306}S_{46}$	6.4%
Seminal plasma protein PDC 109	15480.6	4.91	18	13	$C_{703}H_{1025}N_{175}O_{200}S_{11}$	16.4%
L-lactate dehydrogenase A	36597.6	8.12	35	37	$C_{1638}H_{2649}N_{443}O_{473}S_{15}$	6.0%
Calmodulin	16837.6	4.09	38	14	$C_{719}H_{1129}N_{189}O_{256}S_{10}$	6.7%
Glyceraldehyde 3 phosphate dehydrogenase testis-specific	43288.0	8.32	44	47	$C_{1936}H_{3078}N_{520}O_{567}S_{18}$	7.1%
Outer dense fibre protein 2	75498.1	7.52	115	116	$C_{3223}H_{5360}N_{968}O_{1040}S_{37}$	2.9%
Triosephosphate isomerase	26689.5	6.45	29	28	$C_{1189}H_{1893}N_{327}O_{356}S_{7}$	6.8%
SPACA1 Sperm acrosome membrane-associated protein 1	30938.8	4.55	48	25	$C_{1354}H_{2144}N_{368}O_{436}S_{12}$	6.1%
Tubulin alpha 3	49925.5	4.98	63	40	$C_{2218}H_{3431}N_{593}O_{676}S_{22}$	9.3%
L-asparaginase	32050.4	7.00	34	34	$C_{1382}H_{2258}N_{406}O_{440}S_{14}$	3.6%
Tubulin beta-4B chain	49831.0	4.79	62	37	$C_{2191}H_{3373}N_{593}O_{686}S_{26}$	9.7%
Tubulin beta 4ª	49585.8	4.78	61	36	$C_{2182}H_{3358}N_{590}O_{681}S_{26}$	9.7%
ATP synthase subunit beta, mitochondrial	56283.5	5.15	64	48	$C_{2505}H_{4049}N_{681}O_{762}S_{12}$	5.3%
F-actin-capping protein subunit beta	33741.3	6.01	43	40	$C_{1471}H_{2353}N_{415}O_{463}S_{15}$	6.6%
Tubulin beta 2B	49953.1	4.78	62	37	$C_{2191}H_{3371}N_{595}O_{690}S_{27}$	9.7%
Transmembrane protein 190	19743.3	5.00	25	18	$C_{861}H_{1331}N_{243}O_{263}S_{14}$	8.3%
Keratin, type I cytoskeletal 19	43885.0	4.92	61	44	$C_{1898}H_{3048}N_{546}O_{628}S_{10}$	6.3%
Desmoplakin	332382.7	6.47	467	455	$C_{14381}H_{23449}N_{4207}O_{4622}S_{98}$	5.6%
Elongation factor 1-alpha 1	50140.9	9.10	53	64	$C_{2235}H_{3589}N_{611}O_{659}S_{18}$	6.7%
Seminal plasma protein BSP-30 kDa	21269.2	5.73	22	20	$C_{975}H_{1431}N_{239}O_{274}S_{12}$	16.4%
RAB2B, member RAS oncogene family	24167.4	6.59	26	25	$C_{1059}H_{1668}N_{302}O_{324}S_{11}$	8.8%
Leucine-rich repeat and fibronectin type III domain containing 2	84279.1	6.43	75	70	$C_{3719}H_{5933}N_{1045}O_{1127}S_{30}$	6.0%
Voltage-dependent anion-selective channel protein 1	30839.7	8.82	29	33	$C_{1376}H_{2151}N_{369}O_{428}S_{4}$	10.2%
CLRN3	25407.4	9.55	10	17	$C_{1169}H_{1826}N_{294}O_{328}S_{5}$	12.2%
SCAMP1	39986.8	8.09	30	32	$C_{1828}H_{2739}N_{471}O_{506}S_{18}$	14.1%
Keratin, type II cytoskeletal 5	62623.7	8.21	64	66	$C_{2709}H_{4294}N_{796}O_{881}S_{16}$	7.9%
Carboxypeptidase	66281.2	9.36	51	70	$C_{3027}H_{4651}N_{809}O_{833}S_{18}$	12.3%
Uncharacterised protein	188419.2	8.16	163	169	$C_{8685}H_{13545}N_{2143}O_{2389}S_{69}$	12.1%

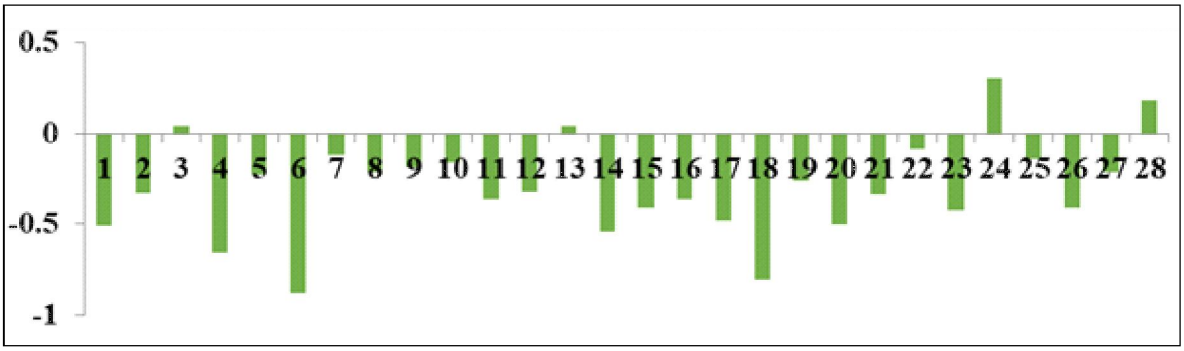


Fig 2: The predicted value of the grand average of hydrapathicity**.**

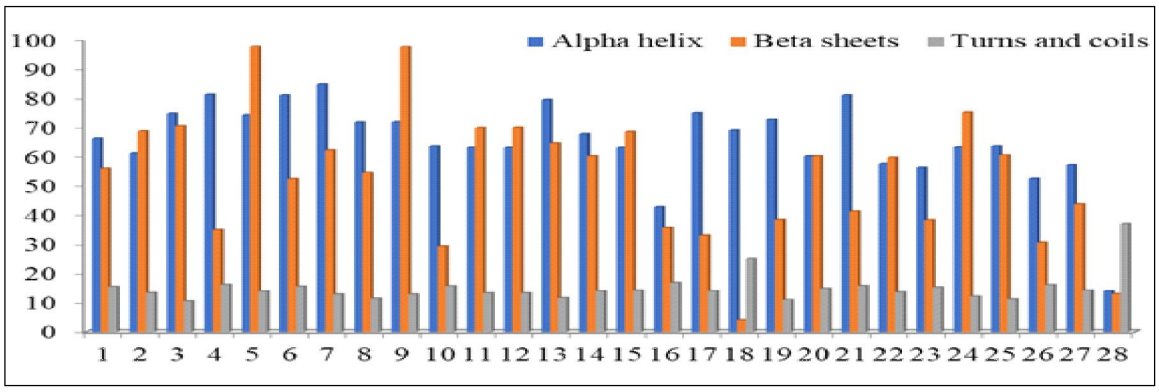


Fig 3: Predicted percentage of alpha helix, beta sheets and turns and coils in the secondary structure of 28 proteins under this study.

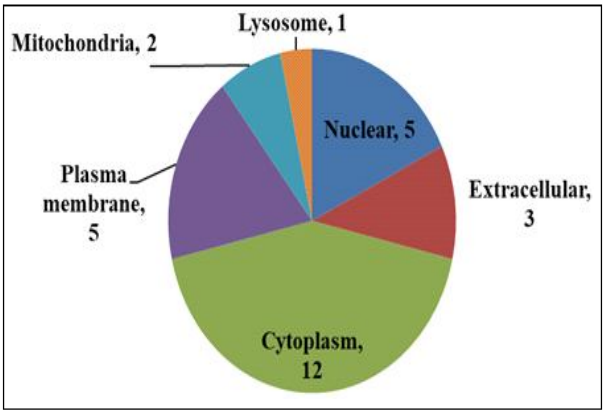


Fig 4: Subcellular localisation of the 28 proteins in this study.

Tubulin beta 2B, Keratin, type I cytoskeletal 19, Elongation factor 1-alpha 1 and RAB2B, member RAS oncogene family were observed in cytoplasm; 5 proteins, *i.e.*, SPACA1, Leucine-rich repeat and fibronectin type III domain containing 2, CLRN3, SCAMP1 and Uncharacterised Protein were located in Plasma membrane; 2 proteins, *i.e.*, Voltage-dependent anion-selective channel protein 1 and ATP synthase subunit beta, mitochondrial were located in mitochondria and one protein, carboxypeptidase is located in Lysosomal region (Fig 4). The proteins that were found to be located in the plasma membrane can serve as a possible candidate for antibody production for the sex sorting of sperm.

Domain characterization of the proteins

For domain analysis of the proteins, the SMART tool was used (Fig 5). The domains are a protein's separate or distinct structural and functional units. They contribute to a specific function or interaction of the protein and are responsible for the overall role of the protein (Letunic *et al.,* 2018; 2021).

Protein-protein interaction

STRING database was used for the prediction of protein-protein interaction. The results obtained showed the interaction of different proteins and their function with a score of their interaction. A higher score indicates a stronger interaction. Nodes represent the proteins and edges represent their interactions. In the network, the red line indicates the presence of fusion evidence, the green line indicates neighbourhood evidence, the blue line indicates co-occurrence evidence, the purple line indicates experimental evidence, the yellow line indicates text mining evidence, the light blue line indicates database evidence and black line indicates co-expression evidence (Snel *et al.,* 2000). The protein-to-protein interaction of the X-specific protein CLRN3 and Y-specific protein SCAMP1 are shown in Fig 6.

Prediction of linear epitopes from protein sequence

The linear epitopes of the proteins (Table 2) were predicted using Bepipred linear epitope prediction version 2.0 on the IEDB server, using the FASTA sequence of each protein.

The BepiPred-2.0 server foretells B-cell epitopes of a protein sequence and it uses a Random Forest algorithm which is taught on epitopes and non-epitope amino acids of crystal structures. The residues that have above the threshold score (0.5) were foretold to be a portion of an epitope and were shown in yellow colour on the graph (Jespersen *et al*., 2017).

Prediction of epitopes based on three-dimensional structures of proteins

The Ellipro tool was used to predict epitopes based on the proteins' three-dimensional structures (Table 2). This is based on a method of flexibility and solvent accessibility. Ellipro uses the protein's three-dimensional structure in

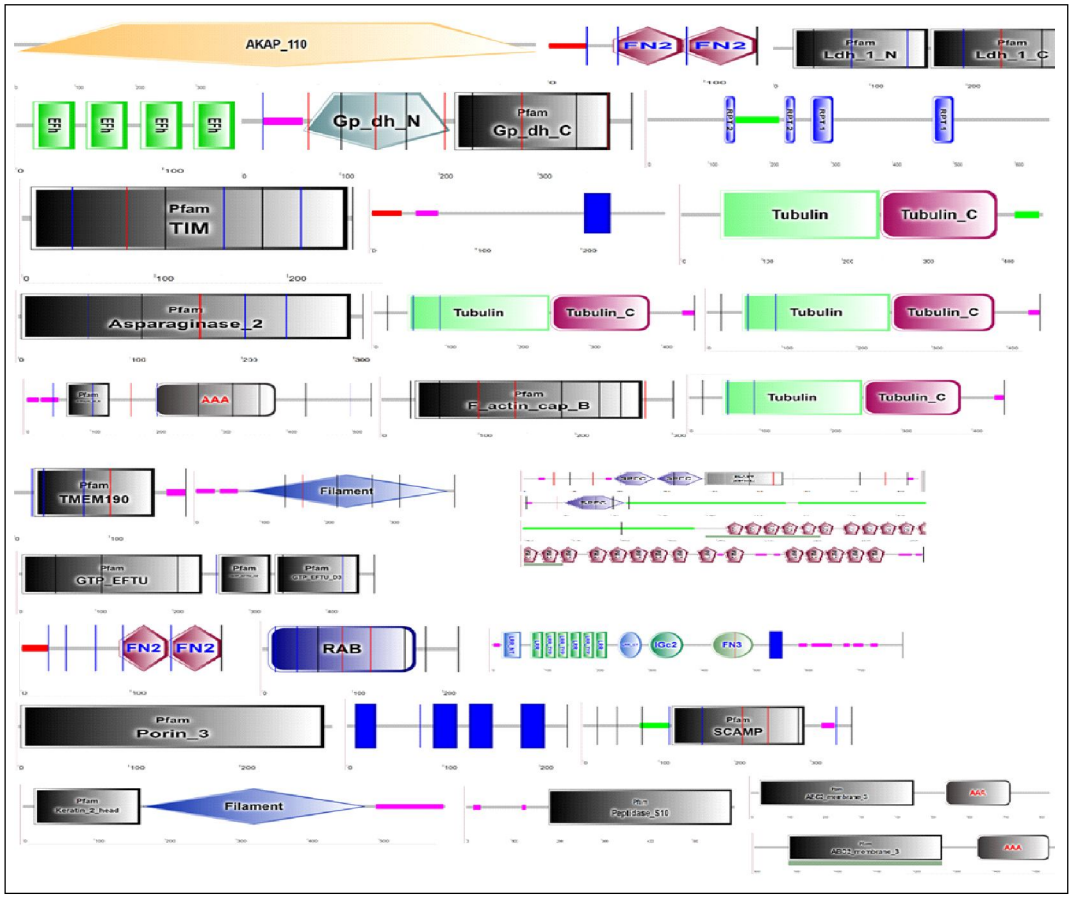


Fig 5: Predicted domains of 28 proteins.

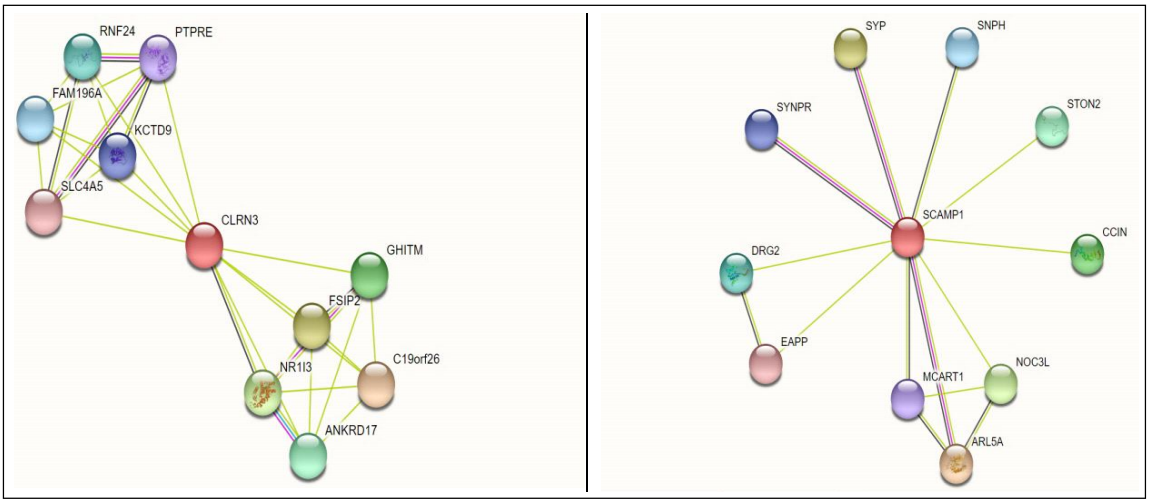


Fig 6: Predicted functional partners of X-specific protein CLRN3 and Y-specific protein SCAMP1.

Table 2: Prediction of epitopes based on three-dimensional structures of proteins.

Name of the protein	No. of predicted linear epitopes	No. of predicted discontinuous epitopes
A kinase anchor protein 3	18	5
Seminal plasma protein PDC 109	4	4
L-lactate dehydrogenase A	14	10
Calmodulin	8	6
Glyceraldehyde 3 phosphate dehydrogenase testis-specific	11	4
Outer dense fibre protein 2	2	8
Triosephosphate isomerase	8	4
SPACA1	9	6
Tubulin alpha 3	12	6
L-asparaginase	10	4
Tubulin beta-4B chain	17	5
Tubulin beta 4[a]	20	5
ATP synthase subunit beta, mitochondrial	22	5
F-actin-capping protein subunit beta	13	4
Tubulin beta 2B	14	5
Transmembrane protein 190	4	3
Keratin, type I cytoskeletal 19	8	6
Desmoplakin	122	4
Elongation factor 1-alpha 1	14	0
Seminal plasma protein BSP-30 kDa	6	4
RAB2B, member RAS oncogene family	7	6
Leucine-rich repeat and fibronectin type III domain containing 2	44	3
Voltage-dependent anion-selective channel protein 1	12	7
CLRN3	6	4
SCAMP1	6	8
Keratin, type II cytoskeletal 5	3	4
Carboxypeptidase	15	5
Uncharacterised protein	51	8

PDB format to predict several discontinuous epitopes on the given structure. The threshold value for the epitopes in this study was taken to be 0.6.

CONCLUSION

The present study has been conducted on 28 deferentially expressed plasma membrane proteins selected based on proteomic profile of the sexed semen of bovines. The *in silico* characterisation such as physicochemical properties, primary, secondary and tertiary structures, membrane topology, sub-cellular localisation, domain analysis, protein interactions and epitope profiling of both linear and discontinuous peptides has been done for all 28 proteins by using different tools and algorithms to find a suitable target protein which would act as a marker for sorting of bovine semen by an immunological technique or to predict an antibody to be used as a means of separation for X and Y bovine sperm. The proteins that have their localization on Plasma membranes like SPACA1, Leucine-rich repeat and fibronectin type III domain containing 2, CLRN3, SCAMP1 and Uncharacterised Protein may act as antigenic protein to be used for further production of sex-specific antibodies and separation of X and Y sperm. Epitope profiling of the proteins, specifically X and Y sperm membrane bovine proteins can further be used for antibody production and synthesis of artificial antibodies.

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Author contribution

Prachi Sharma, Mridula Sharma, Kamal Devlal and Gohar Taj, conceptualized the manuscript. Prachi Sharma and Apoorv Tiwari conducted the *in-silico* experimental analysis. Mridula Sharma, Prachi Sharma and Dinesh Pandey assisted and edited the manuscript. Gohar Taj, Apoorv Tiwari, Shingini Sharma and Mridula Sharma contributed to critically revising the draft and updating the manuscript for publication.

Conflict of interest

Authors declare that there is not any conflict.

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