Review Article

Scope of Immunological Technologies in Sexing of Bovine Spermatozoa

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ABSTRACT

Current semen sexing techniques efficiently sort X and Y-bearing sperm populations, and also provide us an opportunity to discover proteins differences between separated populations of X- and Y-spermatozoa and can further be used in designing immunological methods to sort sperm cells. Immunological sperm sexing is presumed as a fruitful method for the future sperm sorting. The presence of different proteins in the X and Y sperm populations forms the basis of the immunological approach to sperm sexing. Isolated or identified specific proteins of X and Y sperm would serve as a marker, against which antibodies could be developed and will be used for X and Y sperm enriched semen production. Keeping that in mind, this paper is aimed with a brief overview for scope of immunological techniques as well as its amalgamation with bioinformatics, particularly *insilico* studies for the purpose of developing new and efficient methods of semen sexing.

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Keywords: Semen, Sex-specific proteins, Bioinformatics, *insilico*, semen sexing

Introduction

Presently sorting of sperm based on their sex has an incredible scope since by using sexed semen the production of excess male calves can be minimized.Bull slaughter is prohibited in our country and bull management is very difficult in absence of sex sorted artificial insemination. Semen produced by mammalian males, contains nearly 50% sperm with X chromosome and remaining 50% with Y chromosome. Male and female-producing sperms have differences in their size, weight, density, swimming speed, electrical surface charges (a positive surface charge on Y sperm and a negative surface charge on X sperm), surface macro-molecular proteins, linearity and straightness of path etc. Somatic cells in domestic cattle contain 60 chromosomes whereas male gametes contain half of the number. There is a difference in length of the bovine sex chromosomes which is approximately 4.2% more in X sperm compared to Y sperm, specifically 4.98% in Holeisten, 4.24%in Jersy, 4.05% in Angus, 4.05% in Hereford, and 3.73% in Brahman (Garner, 2009).

Semen which has either X or Y chromosome bearing sperms is known as sexed semen and is used to produce offspring of the desired sex that is either female or male with an accuracy of 80–90%. This technology was developed by the United States Department of Agriculture (USDA). Researchers in Livermore, California, and Beltsville, Maryland developed this technique which was patented as "Beltsville sperm sexing technology" (Garner, 2001). The brief literature that has been reviewed for the present article is categorized under subheading as immunological sperm sorting, proteomic profiling of sperm, biotechnological application of proteomics, and bioinformatics analysis.

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Immunological Sperm Sorting

The idea behind the primary concept of immunological methods for sperm sexing is the presence of different proteins on the surface of X- and Y-chromosome-bearing sperm (Seidel and Johnson, 1999). If we can isolate or identify protein marker(s), then possibly we can also develop antibodies against specific surface proteins of X- and/or Y sperm. Different approaches to immunological sperm sexing are:

Sperm Proteins

In addition to nuclear proteins, the sperm cell also contains a huge amount of tail proteins such as tubulin, cytoskeletal proteins, mitochondrial proteins, and acrosomal proteins such as acrosin (du Plessis *et al.*, 2011). The cellular structure of sperm contains the sperm head, mid-piece, and tail. It is quite different from other cells. There are different types of proteins localized on the surface of the sperm. The differentiation of different regions of the surface of the sperm head surface can be done along with their functions in the fertilization process. It has been demonstrated that sperms from the mice in which the Fertilin B protein was knocked out were severely inadequate in binding to the ZP (Evans *et al.*, 1997). Fertilin B is a ZP-binding protein. It is a transmembrane protein that is localized in the sperm head plasma membrane. Then Ensslin *et al.* (1998) observed a protein, P47 which is linked with the sperm plasma membrane.In 1999 Nakamura and his coworkers talked about the highly expressed rhophilin mRNA in adult testes. They did an immunofluorescence analysis that showed that rhophilin is localized in the fibrous sheath of the sperm tail and is expressed in germ cells.

Later it was observed that cAMP-dependent phosphorylation of flagellar proteins is at least partially responsible for the initiation and maintenance of the motility of sperm (Si and Okuno, 1999).A significant number of proteins are also recognized in the sperm tail even

though the sperm head is the chief source of sperm protein. In one study,when the subcellular fractionation techniques were combined with proteomic analysis 1049 proteins were identified by Amaral and coworkers (2013).These proteins were categorized and it was revealed that 26% out of these proteins were related to metabolism and energy production, 11% were related to sperm tail structure and motility, and 24% were associated with lipid metabolism. These results were indicative of the role of proteins from sperm tails in the capability of sperm to attain energy from endogenic pools. A failure in fertilization may occur due to the loss of these proteins (Amaral *et al*., 2013).

Chen *et al*. (2012) have reported that there are 31 differentially expressed genes, out of which 27 were up-regulated in X chromosome-bearing sperm and 4 were up-regulated in Y chromosome-bearing sperm. This difference between X- and Y-sperm can also lead to phenotypic variations in X- and Y-sperm. The differences in DNA between X and Y chromosome-bearing sperm may also give rise to the protein differences between these sperms (Talokar*et al.*, 2017; Yadav *et al*., 2017).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). If we are to isolate or identify different proteins between X and Y sperm then these will serve as a marker, against which antibodies could be developed and this is the basic principle behind the immunological method of sperm sexing.

Sex-specific antigen

If we can identify the sex-specific proteins in X- or Y-sperm then it would serve as a great support to develop an immunological method for sorting sperm cells. In 1999, Blecher and his co-workers did a study in which they tried an enrichment for sex-specific proteins by removing non-specific proteins by the use of antibodies. They did this experiment on rabbits and used affinity chromatography to achieve the separation. They were able to obtain purified sex-specific protein through this method (Blecher *et al*., 1999).Later, many efforts and studies have been made to search for the differences between X- and Y-sperm by using modern techniques like two-dimensional gel electrophoresis. But none of them gave a conclusive result on the presence of sex-specific proteins in the sperm cells (Hendriksen *et al.*, 1996, Yadav *et al.*, 2017). Numerous methods have been tried for immunological sperm sexing but none of them were able to achieve recurrent success (Hendriksen *et al.*, 1996). However, the likelihood of detection as well as separation of sperm cells is related to the antibodies against

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the specific sperm proteins therefore it is very meaningful to try and identify these proteins (De Canio *et al.*, 2014; Yadav *et al*., 2017).

Cell surface antigens

Cell surface antigens that are specific to either X- or Y-chromosome-bearing sperm provide a possible means of differentiating the sperm populations. According to his observations in 1989, Bradley stated that the H-Y antigen may act as a possible way for sorting semen as it is a male-specific protein (Bradley, 1989). This attracted the attention of various researchers. The H-Y antigen is located in the "male" tissues of mammalian species except in the erythrocytes and premeiotic germ cells. It has been speculated that the differences that are present between X and Y sperm might be present in a minute portion of the cell membrane in a negligible quantity and may have a low antigenicity which is not up to the level of detection by 2-DE (Hendriksen *et al.*, 1996; Soares *et al.*, 2008). In an experiment, Ali *et al.* (1990) separated the Y-sperm from the X-sperm by the use of monoclonal antibodies against the H-Y antigen. They found out that out of the total sperm population in the sample around half was able to bind with the antibody. Yet, after the studies by various researchers, the sex ratio of the offspring was not significantly changed after using the semen that was treated with the identical antibody. Furthermore, there is no solid evidence that the H-Y antigen is specifically present on the Y-bearing chromosome (Ohno and Wachtel, 1978).

In other studies, some indirect hints have been found about the existence of different sperm membrane proteins in X and Y sperm cells (Grant *et al.*, 2008; Zhang *et al.*, 2008).Thus, Beerli*et al*. (2008) emphasized the need to search for an alternative technique that could detect these minute levels of sperm proteins and their differences. He also suggested that in this regard an immunological approach could prove to be beneficial for searching sex-specific proteins.Later it was hypothesized that sex-specific proteins are highly conserved Li *et al*. (2011). Li andcoworkers found that X- and Y chromosome-bearing sperm have different proteins on their cell surfaces and the basis of this observation is likely to devise an immunological-based method for the separation of sperm cells.

The sex-specific gene that is a sex-determining region Y (SRY) is limited to Y-chromosome and this encodes for SRY protein. Prasad *et al*. (2010) postulated that if the H-Y antigen on the surface of Y-bearing sperm cells is present because of the expression of the Y-chromosome, then this can be used as a basis to separate Y-sperm from X-sperm. Additionally, when he did his experiments, he found out that under typical conditions H-Y

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antigen exists on the cell membranes of both X- and Y- chromosome-bearing sperm, leading to the conclusion that sexing of mammalian semen by the use of H-Y antibody is not appropriate (Prasad *et al.*, 2010; Yadav *et al.*, 2017).

Proteomic profiling of sperm cells

Chen and coworkers (2012) conducted a study on semen ejaculates from 9 bulls and found that Actin like CAPZB, tubulin alpha 3, and tubulin beta 2, and actin-binding proteins profilin-beta actin were proteins that were expressed differentially and had an important role in cytoskeleton structure. More levels of beta 4B and tubulin alpha3 isoforms were present in X-cells compared to Y-cells when differentially analysed using 2DE/MS. Then in 2014,Canio*et al*. observed two proteins were upregulated in Y- spermatozoa while 15 were upregulated in X- spermatozoa. They further found that there was more concentration of cytoskeleton proteins in X- sperm populations. These proteins were A-kinase anchor protein 3, 5 tubulin isomers, and 3 outer dense fiber constituents. Four different proteins were over-expressed in X-chromosome-bearing sperm. These proteins were calmodulin (regulatory protein), L-asparaginase (amino acid catabolism), a protein associated with sperm acrosomal membrane, and seminal plasma protein PDC109. These proteins were found to be more abundant with a fold change of X chromosome-bearing cells.

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Separation of sperm proteins

As early as 1874, Miescher was the first one to isolate the elementary component of the sperm from the semen of a salmon. He found out that the amplest nuclear protein in the sperm was protamine. The contamination of the sample, during sperm proteomic analysis, with other proteins will disturb the precision of the analysis. Therefore, the sperm is purified through density gradient centrifugation or the swim-up method to remove or minimalize this type of protein contamination (Sakkas *et al.*, 2000).After the purification of sperm cells, the sperm proteins are analyzed by methods that are based on electrophoretic or chromatographic characterization of proteins tailed by the methods of protein sequencing. An important approach, which is used to identify various proteins from diverse types of cells is two-dimensional gel electrophoresis (2DE) and most of the proteins were primarily separated by using this technique. When used with western blotting analysis, the 2DE technique led to considerable developments in the study of the composition of sperm and its function. The advantages of 2DE and its associated methods are that they are operated by most laboratories and the data that is obtained from 2D maps can be effortlessly understood (Rabilloud, 2002).

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A study in 2002 using dissimilar methods to separate proteins of human cilia showed different results. The Analysis was done by using two-dimensional (2-D) PAGE along with trypsin digestion liquid chromatography/tandem mass spectrometry (LC/MS/MS) sequencing and 38 potential ciliary proteins were obtained. Axonemal proteins were separated using one-dimensional gel electrophoresis to identify ciliary components that were not resolved by 2-D PAGE. An additional 110 proteins were obtained by analyzing with LC/MS/MS in which the gel lane was divided into 45 separate slices Lastly, the preparations of isolated axonemes were processed with Lys-C, and the subsequent peptides were run directly by LC/MS/MS or by multidimensional LC/MS/MS which led to the documentation of further 66 proteins (Ostrowski *et al.*, 2002). Before isolating sperm proteins by using different methods, the purification of motile sperm in the semen sample is also a vital step. Various cellular components are present in the fresh semen, as present in epithelial cells and blood cells. Further, if we consider the cryopreserved semen, it contains egg yolk and milk proteins (Amirat *et al.*, 2004). For further confirmation of contamination of the sample by non-sperm cells specific markers like CD 45 are used (Lambard *et al.*, 2004).

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Hu *et al.*, (2005) emphasized that the output ability of 2DE is lower when it is compared with the methods that rely on the separation of peptides by the use of liquid chromatography (LC). For instance, 10–200 proteins can be identified by 2DE approaches per the study, however, LC-MS/MS approaches can identify thousands of diverse proteins in a comparatively short time. A number of studies have verified that unique results are achieved by different approaches and hence for obtaining dependable results, other precise approaches must be used. For example, it was often seen that the rate of failure was high in getting all the proteins when they were extracted from the cells using the standard methods. Particularly the solubilization of multi-protein complexes is only possible by using aggressive procedures (Tan *et al.*, 2008).

Sperm proteome and sex control

Sex control has a significant application possibility in animal reproduction. Sex selection can be attained before the insemination of the animal through the separation of X and Y-sperm based on the unique characteristics of their DNA. The initial sorting of X and Y chromosome-bearing sperm was done with the help of a modified jet-in-air flow cytometer (Johnson *et al.*, 1989). Up to now, the flow sorting technology has been used as a commercial method for sperm sexing for numerous breeding purposes and thousands of pre-

sexed progenies in a huge number of species have been produced (Abeydeera*et al*., 1998; Hamano *et al*., 1999; O'Brien *et al*., 2003; Gao *et al*., 2009). However, reduced fertility with high embryo mortality, retarded embryo development, and reduced litter size have been observed since the first progeny was obtained by the use of sex-sorted sperm. It has been described that sex-sorted sperm shows decreased practicability and contradictory membrane status in comparison to non-sorted conventional sperm. This might affect capacitation or acrosomal reaction due to the sorting process (Gillan *et al*., 2005). Recent advancements have shown that an increased dilution of sperm, exposure to laser light, and physical stress during the processing of sperm through the flow cytometer may serve as a major reason for the reduction in fertility of sexed sperm (Gillan *et al.*, 2005). Due to the reduction in fertility of sexed sperm which are sorted using flow cytometry, it is essential to develop more efficient, cheaper, and faster methods for the production of sexed semen. In-depth knowledge of sperm biology will serve as the basis for the development of new technology in semen sexing. In recent years, increasing pieces of evidence have shown that X and Y chromosome-bearing sperm express different proteins, which provide a novel way to distinguish sperm with the help of immunological methods (Sang *et al.*, 2010; Alminana ˜ *et al.*, 2014).

Interestingly, a specific protein was identified in the X chromosome of a mouse but there was no proof that this specific protein was linked to sex control (Yeh *et al.*, 2005). de Graaf *et al.* (2006) showed that sex-sorted ram sperm was released more readily from epithelial cell monolayers of the oviduct as compared to non-sorted sperm. Later in 2010 Sang *et al.* designed a study to identify sex-specific antibodies. The study showed that X or Y sperm can induce different antibodies in the serum of rabbits, and a 30 kDa protein was found to capture X-sperm. Some of the sex-specific proteins have been explicitly identified in X or Y chromosome-bearing sperm. The sry gene encodes for sex-determining region Y protein (SRY) and it is specifically present in the Y chromosome. Both proteins of SRY and its transcript have been found in Y chromosome-bearing sperm (Li *et al*., 2011a; b).

Moreover, a piece of indirect evidence has shown that diverse proteins are present in the X or Y-chromosome-bearing sperm. Because of that X or Y-chromosome-bearing sperm can certainly inflict sex-specific transcriptomic responses in the oviducts. These indications propose that different proteins are located in the membrane of X or Y-chromosome-bearing sperm (Alminana ˜ *et al.*, 2014). Progressively different proteins amongst X and Y sperm have been identified recently with proteomic technology. It was found, in two different types

of sperm cells from the bull, that the differential expression of proteins is related to the structures of the flagellar cytoskeleton (De Canio *et al.*, 2014). One more study detected 42 note-worthy protein spots in X and Y chromosome-bearing sperm. It was found that these proteins are related to stress resistance, the activity of serine proteases, energy metabolism, and cytoskeletal structure. According to the results observed, we can suggest that these proteins may affect the binding and fusion of sperm with the oocyte, and its functions along the development and growth of the embryo. In the future, there can be development of new techniques to separate X or Y-chromosome-bearing sperm using these differentially expressed proteins that are localized in the sperm membrane (Chen *et al.*, 2012).

A sex-specific antibody has been used for the immune precipitation of sex-specific proteins in the sperm of bovines, after analysis with two-dimensional gel electrophoresis. 52.1, 15.2, and 7.6, 15.2, and 52.1% of unsorted sperm, sex-sorted X-sperm, and sex-sorted Y-sperm were obtained and recognized by the neutralized rabbit antisera. The purity of the process of separation of sperm cells using supposed X-sperm sex-specific antibodies reached 74.3% when they were immunologically separated and the sperm was introduced into the oocytes This resulted in providing a theoretically more effective way for separating the two sperm populations and laid a foundation for forthcoming research for the search of sex-specific proteins (Yang *et al.*, 2014).

Hendriksen *et al.* (1996) examined the boar sex-sorted semen using 2-dimensional SDS-PAGE but failed to detect any significant differences between the two sperm populations.Howes*et al.* (1997) took ejaculates from 4 bulls and then did SDS-PAGE in protein (detergent extracted). They were stained using Coomassie blue after being resolved by a non-reducing SDS PAGE. The study failed to reveal any significant difference between the protein profiles of the four samples. They further did radio-labeling of the surface protein of the sperm cells with Iodogen reagent and Bolton and Hunter reagent and performed a FACS separation but did not get any satisfactory results depicting differences between X- and Y- sperms.

A 50 kDa protein was observed in the blots of unsorted and Y-chromosome-sorted spermatozoa was observed by Howes *et al.* (1997). However, when the experiment, no difference in sperm surface protein was detected through one-dimensional SDS-PAGE and immunological methods. When 2 dimensional SDS PAGE was performed, a 40 kDa X specific bandwas found to be absent but probably it was an intracellular protein (Howes *et*

al., 1997).Yeh *et al.* (2005) observed proteins like ESX1 and SRY that were detected specifically in the Y chromosome. Sang *et al*. (2010) found X-specific 30kDa protein which was immune-precipitated by using rabbit sera against X chromosome-bearing sperm. Scott *et al*. (2018) used the SWATH technique, to identify 8 differentially expressed proteins between Y and X spermatozoa. They found axonemal (DNAI2) and dynein intermediate chain 2 protein which is ampler in Y- sperms. FUNDC1 and FUNDC2 wereother proteins of bull semen which was found in more concentration in the X-sperm population, mitochondrial protein (NDUFS7-complex I) and NADH dehydrogenase iron-sulfur protein 7 were other proteins found to be differentially expressed. They observed that most of the differentially expressed proteins had their functions in cell metabolism or energy production.Sharma*et al*.(2022), in their study on Sahiwal semen, reported that at about 18 – 24 kDa and 30-37 kDa strong bands were seen in the X-sorted sperm population, which were absent in the Y-sorted population. Y- specific band was also observed at about 25KDa. Quelhas*et al.* (2021) reported fifteen differentially expressed plasma membrane proteins in X and Y bovine sperm, out of which 12 were found to be abundant (up-regulated) on X spermatozoa and 3, were abundant (up-regulated) on Y spermatozoa. Out of these 15 proteins, they concluded that a 34kDa protein, sperm acrosome membrane-associated protein 1 (SPACA1), was the best candidate for developing an antibody-based X and Y sperm separation method.Shen*et al.* (2021) studied plasma membrane proteins of X and Y chromosome-bearing sperm of bovine semen from 20 different Holstein Bulls. 1521 proteins were identified in total. They also found that CLRN3 is specifically expressed on X spermatozoa and SCAMP1 protein was specifically expressed on Y spermatozoa.Laxmivandana*et al.* (2021) studied differential proteins associated with X and Y sperm plasma membranes and found 470 different proteins associated with the plasma membrane of bovine sperm. They further noted 13 differentially abundant proteins in X and Y-sorted sperm, out of which 2 were down-regulated in Y sperm but not in Y sperm, 4 were abundant/found on X sperm and 7 proteins were found/abundant on Y sperm and not in X sperm.

Bioinformatics Analysis of sperm Proteins

To understand the complete workings of a cell, along with the molecules and processes that are completed inside Bioinformatics, various studies were carried out which promoted the use of different sciences and disciplines to enable the advancement in its development and characterization. The sequencing of organic molecules began in the 1950s. The sequencing of the first protein was done in 1956 and Margaret O. Dayhoff discovered that an enzyme i.e.,

bovine insulin, is a small peptide having fifty-one amino acids (Dayhoff, 1969). The first database for biological sequences was created in 1965. It stored the protein and DNA sequences that were defined till that time and made them accessible to the community of researchers. After eight years of this development, the first known database was created, which is widely used even today, Protein Data Bank (PDB) (Bernstein *et al*., 1977). By the 1980s, bioinformatics had emerged as a new tool for scientific research. PIR (Protein Information Resource) and Swiss-Prot are the protein sequence databanks and were formed between 1987 and 1990. One of the most important landmarks in bioinformatics, the BLAST algorithm (Basic Local Alignment Tool) originated in 1990. This tool entirely transformed the investigation and exploration of biological sequences in the databases (Altschul *et al*., 1990).The National Institute of Health of the United States described bioinformatics which can also be called computational biology which trades in the advancement and implementation of theoretical methods and analytical data, computer simulation techniques, and mathematical modulation to study biological, social, and behavioral systems (Quíceno, 2006). The National Center for Biotechnology Information (NCBI) has given the following definition of bioinformatics, "Bioinformatics is a department of science in which numerous subjects such as statistics, applied mathematics, biochemistry, chemistry, artificial intelligence, computing, and information technology unite, whose aim is to enable the innovation of new biological concepts, as well as generate global outlooks from which merging of the principles in biology can be recognized" (Meneses-Escobar *et al*., 2011).

Applications of Bioinformatics

By reviewing the various projects of bioinformatics it has been established that the necessities of this particular field need the storing of huge amounts of data, with numerous dimensions, of lengthy periods, and with diverse formats along with their sources. It emphasizes giving structural and chemical information for tiny molecules. Keyword-based queries are accepted and a graphical interface is also provided for direct searches of chemicals, and thus access to an extensive range of web resources is allowed (Judice *et al*., 2005).The fields in which bioinformatics is presently being developed are numerous and diverse. They range from the acquisition of DNA data directly or assays of protein sequences (by using techniques like mass spectrophotometry), to the formation of software with the purpose of analysis and storage of the data, which suggests the development of algorithms that need both biological and mathematical knowledge. Bioinformatics finds its use in the

areas of proteomics, genomics, phylogeny, and pharmacogenetics (Bustos *et al.*, 2011).Bioinformatics includes the growth and development of databases for storage and retrieval of biological data, algorithms to evaluate and find their associations with biological data, and lastly, statistical tools to recognize and understand the data sets (Hernández-Domínguez *et al.*, 2019).

Proteomics data analysis

Proteomics encompasses numerous technologies for thorough proteome analysis and thus accomplishing identification and quantification of the proteins. It covers interaction studies, analysis of genetic products functionally, and localization of proteins, which in turn helps in explaining the protein identity of a creature to recognize its function and structure. Yet, seeing that the proteome is extremely dynamic because of the complicated systems of regulations that regulate the amounts of expression of proteins, its usage is restricted, Subsequently, on top of the use of specific facilities, personnel, and types of equipment, the software is also encompassed for databases and equipment, which upturns the costs (Pandey *et al.*, 2000; Seaton *et al.*, 2018; Yanovsky and Kay, 2002). Proteomics is continuously being updated but has obstacles that range from the preparation of samples to the collection of data. A huge quantity of data is produced by the prediction of unidentified protein structures and their functions, three-dimensional structures, protein folding models, and data which is attained from the differentiation of proteins in two-dimensional gel electrophoresis, peptide mass fingerprinting (PMF), 2D protein visualization isoelectric focusing, etc. All of these generate proteomes with high performance through the incorporation of bioinformatics, which presents novel algorithms to handle a huge quantity of dissimilar data (Blueggel*et al.*, 2004; Popov *et al.*, 2009; Schmidt *et al.*, 2014).

All these data are of enormous assistance, as it is being used in diverse areas of research, such as identification of contenders for production of the vaccine, diagnostic markers, understanding the pathogenicity mechanism, understanding of functional protein pathways regarding their role in various ailments, and modification of patterns of expression as a response to numerous signals (Aslam *et al.*, 2017; Chaudhary *et al.*, 2019; Yatoo*et al.*, 2019; Burgos-Canul *et al.*, 2019; Stroggilos*et al.*, 2020).Some of the commonly used platforms in proteomics are ExPASy (Expert Protein Analysis System), BLAST (The Basic Local Alignment Search Tool), and Protein Data Bank (PDB). BLAST is amongst the most updated and most commonly used platforms. It uses modest but potent approaches for the

analysis of protein for the comparison of amino acid sequences. This makes it likely to find out the homology amongst the proteins. The algorithms that are used to accomplish this process assure the best conceivable alignment (Needleman and Wunsch, 1970; Altschul *et al.*, 1990; Smoot *et al.*, 2004; Barton, 2007; Johnson *et al.*, 2008; Popov *et al.*, 2009). ExPASy provides access to varied analytical tools and databases that are devoted to proteomics and proteins. Alternatively, PDB is the worldwide source of 3-dimensional structures of macromolecules. It is weekly updated and comprises more than 153,000 structures of proteins, derived from X-rays, crystallographic studies, or nuclear magnetic resonance (NMR) which is made by modeling software. All of these platforms hold numerous servers that help in the classification of proteins based on their structure, sequence, and function (Popov *et al.*, 2009, Gasteiger *et al.*, 2005; Rose *et al.*, 2010).

Phylogeny in the Protein Evolutionary Process

The genome sequencing of organisms has permitted to recognition of the set of all of their genes, revealing the functions and the products expressed by them, along with the regulation mechanisms in various processes of metabolism, where a boundless number of proteins takes part. (Marcotte *et al.*, 1999). Phylogenetic profiles, for the past few years, have been important to link homologous proteins by sequence alignment. It has been discovered that numerous proteins share conserved regions and related structures (Pellegrini *et al.*, 1999). Phylogeny studies the alterations that happen inside the sequences and assembles them in a chart with results which is called a phylogenetic tree, All the sequences that are of the same family can be put together into a clade and then into subfamilies which provides the data on the functional diversity and evolution (Song *et al.*, 2018).

Due to Evolutionary mechanisms, homologous protein families have arisen, which have a shared ancestor (Kaminska *et al.*, 2009). To evaluate these alterations in the sequences, bioinformatics programs make use of mathematical models and algorithms, established on experiential matrices of amino acid replacement, along with those that combine properties of the structure in the native state, for instance, secondary structure and approachability (Bastolla and Arenas, 2019). The phylogeny studies of proteins at this time are needed to distinguish protein-protein interactions within the biological systems. Structural or molecular analysis of proteins will call for more data to counter if a protein exists in one or numerous species and also to foretell the evolution times and common ancestor (Szurmant and Weigt, 2018).

Insilico studies

In biology and other experimental studies, an *insilico* experiment is performed on a computer or by using computer simulation. Tools for *Insilico* analysis of protein are of major significance for making use of data for the development of drugs and health care. Proteins are complex macromolecules that have an essential role in cellular activity. They increase biological processes or reactions by the expression of catalytic activity. Thus, analysis and knowledge of protein are substantial for determining the protein function and understanding its role and mechanism in an organism's body (Pruess and Apweiler, 2003; Singh *et al*., 2016). In 2021 Sharma performed *insilico* studies on characters such as physicochemical properties, primary, secondary, and tertiary structures, membrane topology, sub-cellular localization, domain analysis, protein interactions, and epitope profiling, both linear and discontinuous peptides was done for 28 differentially expressed plasma membrane proteins by using various bioinformatics tools and software. She concluded that by using *insilico* studies localization of proteins can be confirmed and particularly plasma membrane-specific differential proteins can further be used to produce artificial antibodies through epitope profiling which can pave the way for immunological sexing of spermatozoa.*Insilico* study can prove to be an essential tool to guide the research for the development of an immunological method of sperm sexing.

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Conclusion and Future Prospects

A detailed study on proteomic profiling of X and Y-sorted sperm and then analysis of such differentiallyexpressed proteins and finding antibodies against those sex-specific and differentially expressed proteins is the most essential step to look forward to the immunological sexing of sperm. By *insilico* analysis one can successfully predict the location, domains, interactions, structures, epitopes, and binding of various molecules or antibodies to theprotein being studied. By assessing the differentially expressed proteins *insilico*, 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. 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 promising immune sexing technique for spermatozoa.

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