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Review Article

SERUM PROTEOMICS IN EARLY PREGNANCY DIAGNOSIS OF BOVINE: A REVIEW

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Abstract: Early pregnancy diagnosis in bovine is yet to be addressed by the modern scientific communities across the globe. Latest tools available to diagnose pregnancy in bovine particularly cattle and buffalo are not completely definitive. Farmers of present era are in great anticipation on modern scientific researches and technologies to get relief from this long-standing problem. It has been augured that recent advances in proteomics, especially in serum proteomics may bring a break through to develop a new tool to solve this challenge by discovering novel biomarker(s). This review emphasized the most promising technological approaches toward decrypting the serum proteome and exertion of the knowledge in reproductive biology would bring an end to the chronic problem of early pregnancy diagnosis by leading to the discovery of novel pregnancy biomarker(s).

Key words: Serum proteome, Pregnancy diagnosis

INTRODUCTION

In the livestock sector, cattle and buffalo plays an important role in the growth of India's economy. To maintain or to increase the economic pace of our country from livestock sector high reproductive efficiency and management of large animals, especially cattle and buffalo is pre-requisite. Early pregnancy diagnosis or early identification of non-pregnant animals post breeding improves reproductive efficiency as well as profitability of commercial dairy farms. Because economic loss is mostly found in dairy or beef animal farms owing to improper early pregnancy diagnosis. Moreover, in case of buffalo the early pregnancy diagnosis is more problematic as they are silent estrus animals and needs urgent attention. Tools that are available for early pregnancy diagnosis have disadvantages in one or other way. Early pregnancy diagnosis has now become a basic and big challenge to the farmers of all categories, from backyard to commercial.

The fundamental aim of early pregnancy diagnosis is to detect non-pregnant animals as early as possible and rebreed them by adopting suitable method to maintain optimum dry period and calving interval. Palpation by per rectum is one of the most accurate method of early pregnancy diagnosis and is routinely used to determine pregnancy status in dairy cattle and buffalo; however with this method it is difficult to detect accurate pregnancy status earlier than 32 to 35 days after artificial insemination [1]. Transrectal ultrasonography can be used as pregnancy diagnosis tool, but only after 30 days exact result can be obtained [2] and requires an expert, skilled technician with sophisticated instruments. Both these methods diagnose pregnancy relatively late as maternal recognition of pregnancy, occurs between days 14 and 18 [3, 4]. Other indicators of early pregnancy includes early pregnancy or conception factor (EPF) [5, 6] and pregnancy-specific protein B (PSPB), popularly known as, pregnancy associated glycoproteins (PAGs) [7,8].

Early pregnancy factor as a marker for early pregnancy has been problematic [5,6] because, EPF bioassay was based on the inhibition of rosette formation which is time consuming and has limited use in routine pregnancy testing in farm animals [9]. PSPB can accurately detect pregnancy from day 30 [10], however PSPB has an absolute long half-life and remains in maternal circulation for long period of 70 days after parturition[8,11] , thus limiting its use as a marker for early pregnancy. Now-a-days, for early pregnancy diagnosis, pregnancy associated glycoproteins (PAGs) ELISA kits are getting popularity which has a peculiar advantage of detecting pregnancy within 25 days [12] in maternal blood after artificial insemination but tendency to give false positive result in post-partum animals has limited its use. Detection of the steroid hormone viz. progesterone, can also be used to determine pregnancy status [13-15]because most oestrus cycles with an average of 21 days, non-pregnant animals at the time of estrus, progesterone concentration is expected to be low, which discriminate them from pregnant cows that have higher progesterone concentration. However, non-pregnant cows may also have elevated progesterone in some cases at the time of estrus like due to persistent corpus luteum in certain pathological condition thereby giving false positive result.

Unlike human being, cattle and buffalo does not produce bovine chorionic gonadotropin for early diagnosis of pregnancy. Therefore, it is expected that monitoring of changes in serum proteome profile from the day of fertilization to earlystage of gestation can lead to discovery of potential biomolecules that might be novel and specific to the physiological status of the animal [16]. Any candidate molecule to qualify as a marker of pregnancy should be least affected by non-animal factors like feeding, environment, drug interaction etc., present in easily accessible body fluids like serum, milk, urine, vaginal discharge etc. and it must be expressed for optimum period so that its presence can be detected accurately [16]. The advancement of post genomic technologies like proteomics during the last decade and its expected appreciation in varied areas of biological science is going to aid the present era scientist to analyse several protein components in complex mixture of protein in body fluids like serum, plasma, milk, urine etc. It is anticipated that proteomics could be the key solution to this problem. Proteomics allows large scale study of protein expression, protein-protein interaction or post translational modification [17]. So it can help us to identify new and unexpected changes in the protein expression, interaction or modification as a result of changed physiological state like pregnancy, other pathological conditions etc. Thus, proteomic based study of pregnancy related protein is a promising area for animal scientist to find novel proteins which can be utilized for developing tools for early pregnancy diagnosis and other allied sectors.

Proteomics in pregnancy diagnosis: Proteomics is the analysis of complete complement of proteins which includes identification and quantification of proteins, differential expression, post-translational modifications, protein-protein interactions, determination of their localization, and, ultimately, their function [18]. Proteomics is expected to bridge the gap between genome sequence and cellular behaviour because genome can predict a probable situation but proteome reveals the real situation.

Approaches for serum proteomics: Basically, there are two approaches for serum proteomics. They are Gel based approach and Gel free approach. In gel based approach polyacrylamide gel is used for separation of proteins whereas in case of gel free approach no such gel is used for protein separation instead some other chemicals and technologies are used to do the same.

Gel based proteomics: Classical proteomics approach evolved as soon as Laemmli reported SDS-PAGE in 1970 and subsequent report of two-dimensional gel electrophoresis (2-DE) method in 1975 by O'Farrell [19,20]. In this approach polyacrylamide is used to prepare gel and different protein molecules are separated by isoelectric focusing on the basis of isoelectric point (pI) and by polyacrylamide gel electrophoresis on the basis of molecular weight, both are collectively known as 2-dimensional electrophoresis [20]. Then differentially expressed protein spots are identified with commercially available software and finally quantification and identification of the spots is done in Mass spectrometry. Strategy for serum proteomics include the following steps:

a) Sampling: To identify certain novel and potential early pregnancy protein biomarker blood sample should be collected from experimental animals at definite interval after post AI such as 0, 7, 14, 21, 28 etc.days for pregnant group and at day 0, 7, 14, 21

etc. for non-pregnant group considering day of estrus as day 0. Serum should be separated and stored at-20 to -80°C till used after fortifying with protease inhibitor.

b) Preparation of serum samples for 2-DE: Sample preparation is one of the key step in order to achieve success in proteomics research because the more pure the protein sample the more likelihood of getting authenticate result.We know that serum is a complex mixture and there are many high abundance proteins like immunoglobulin, albumin, etc., which may mask the presence of other vital low abundance protein that could be a potential biomarker. So depletion of these proteins are utmost important. They can be depleted by chromatography or by using some commercially available kits. Other inhibitory factors present in serum are salts, lipid etc., so all possible methods should be used to get pure protein samples. Separation of protein in isoelectric focusing depends on its pure so strategies should be taken to obtain most probable pure sample.

c) Quantification: Inorder to prevent bias in any proteomics research quantification of protein in sample is one of the major steps. It can be done by any of the available method like Bradford assay, 2-D Quant Kit etc.

d) Isoelectric Focusing(IEF): It is the first dimension separation of proteins. Here proteins are separated on the basis of the isoelectric point. After purification serum samples are rehydrated on Immobilize pH gradient strips (IPG strips)for 16 hours and then they are run on IEF for 5-8 hours depending on length of the strips and purity of the samples. After completion of IEF, IPG strips can be stored for long time at -80°C.

e) Sodium dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE): It is the second dimension separation of protein molecules. In this protein molecules are separated on the basis of molecular mass. It takes around 2-3 hours. Before running SDS-PAGE, IPG strips are equilibrated on equilibration buffer for denaturation and reduction of protein molecules. For sharp separation of low molecular weight proteins Tris-glycine SDS-PAGE can be used. Now-a-days, a technique known as Two-dimensional Difference Gel Electrophoresis (DIGE) [21] is used by many scientist community to decrease the number of technical replicates/gel replicates and load of labor. It allows detection and quantification of proteins between different biological samples within one single gel. Moreover, it has the advantage of requirement of very low amount of protein.

f) Staining of gels: Basically protein molecules are stain with coomassie blue but silver staining is performed when quantities of protein samples are low.

g) Image acquisition, analysis and spot picking: After staining images of the gel are acquired in scanner and analysis of protein spots are done with the help of commercially available software like PDQuest, Image Master 2D Platinum etc. Protein spots with differential expression like over-expressed, under-expressed or newly expressed are identified and picked either manually or with spot picker. And finally spots are digested with trypsin or other reagents for Mass spectrometry.

h) Mass spectrometry: Mass spectrometry (MS) is one of the indispensable and versatile tools in any proteomics experiment. It is basically used for identification and characterization of proteins after protease digestion and ionization [22].Two techniques matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) have paved the way for the modern proteomics. It is beyond the scope of this review to outline the differences in the ionization processes, analysis and the modern mass spectrometers; still a blink of it is briefly described. In MS, basically trypsin digested protein molecules are introduced to the mass spectrometer through electrospray ionization in electro-spray ionization-MS, or laid down on a series of small spots for later mass analysis using matrix-assisted laser desorption ionization (MALDI) whose absolute masses can be accurately measured. These masses are then compared to databases containing known protein sequences or even nucleotide sequence by translating it to protein sequence, and calculate the absolute masses of the peptides from each protein. The results are statistically analysed to find the best match. Mass spectrometry can also be used to quickly sequence peptides, for identifying unknown proteins. Sequence information of peptide is extracted using a technique called MS/MS or tandem MS,

i) Data analysis: It is one of the most crucial steps in proteomics. As mentioned earlier, the mass of a peptide obtained in MS is used as input in a search engine database to predict masses that would arise from digestion of a list of known proteins and finally the best match result is used to identify the proteins. The different database search engines used in proteomics are MASCOT, OMSSA, and SEQUESTetc.

Gel free Proteomics: Unlike gel based, in this approach polyacrylamide or any other kind of gel is not used. According to the research experiment,basically samples are prepared and digested with protease, and there after labelled with certain tags like isobaric tag for relative and absolute quantitation(iTRAQ) [23],tandem mass tags (TMT or TMTs) [24] etc. After that the sample is directly injected to MS through different types of liquid chromatography (basically LC-MS) and analysis is done in a single experiment. The complete work flow for this approach in reference to iTRAQ as labelling tag the full experiment is performed as following.

In the same manner, like for gel based approach the serum samples are collected, prepared and equal quantity pooled from different groups and days of pregnancy and non-pregnancy. ii) Each sample is denatured, reduced and cysteine amino acids are blocked. iii) Proteins are digested with trypsin. iv) Peptides are labelled with different tagging reagent of iTRAQ. v) Then peptide samples are separated by multidimensional chromatography basically at first by strong cation exchanger then by liquid chromatography. vi) Separated fractions are injected to MS most commonly to MALDI-TOF-TOF and proteins are identified. vii) Quantification and data analysis is done for differential expression on the basis of intensity of reported ion using software like ProQUANT, Multi-Q etc.

Validation: The finding of every research experiment is proved by validation. In serum proteomics the basic tool to validate an experiment is Western blotting, Immunoblotting, ELISA etc. Any biomarkers discovered through proteomics approach can be better validated by these techniques because biomarkers are protein in nature and easy to develop antibody against it which helps to develop immunological kits that can be commercialized easily.

Achievements of proteomics in pregnancy diagnosis: Proteomics in animal research is in its babyhood, but still there is some limited documentation on the bovine proteome in relation to pregnancy. In 2005, Jin performed proteomics analysis of serum samples of Holstein dairy cattle at 21 and 35 days after AI of pregnant and non-pregnant and reported profiles of proteins involved in early pregnancy and suggested the potentiality of these proteins to detect early pregnancy in bovine. Out of all the potential pregnancy-specific proteins identified were IgG2a heavy chain constant region, transferrin, immunoglobulin gamma heavy chain variable region and albumin [25]. Further, proteomics of milk samples from pregnant and non-pregnant cows revealed 16 protein spots, out of which 14 pregnancy-specific spots were up-regulated and 2 spots down-regulated in the pregnant milk sample [26]. Pregnancy-specific proteins were identified as IgG1 heavy chain constant region, serum albumin precursor, epithelial keratin 10, conglutinin precursor, and kelch-like ECH-associated protein. Although some of the identified proteins are abundantly present in milk or serum, their molecular weights and pI values were different from normal milk or serum proteins suggesting that subunits or fragments of albumin and IgG may be pregnancy-specific having potential for pregnancy detection [26]. Proteomics analysis of serum of pregnant and non-pregnant buffaloes in early pregnancy elicited that synaptojanin-1, apolipoprotein A-1, Keratin 10, apolipoprotein and Band Von Willebrand factors were differentially expressed in between the groups [16]. In a recent study, it was found that conglutinin precursor, modified bovine fibrinogen and IgG1 were up regulated whereas proteins like hemoglobin complement component 3, bovine fibrinogen and IgG2a were down regulated in the sera of pregnant cattle in comparison to non-pregnant group [27]. In the urine of early pregnant cows, proteins such as mannan-binding protein, insulin-like growth factors and serpin were found to differentially express against the urine of non-pregnant cows [28]. As the identified proteins from different studies are documented proteins having role in embryogenesis and early pregnancy in one or other way, so, they could be promising bio-marker in development of pregnancy diagnostics in bovine.

CONCLUSIONS

There are many challenges in serum proteomics but still it could act like a bar magnet for the scientific

community and could be a promising area to find some potential biomarkers which may lead to development of a very cheap kit for diagnosis of early pregnancy in bovine. Thus, indirectly it may be very helpful to the farmer's community to overcome from heavy economic loss due to miss diagnosis of pregnancy in early stage.

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