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PREFRACTIONATION STRATEGIES FOR BOVINE PLASMA/SERUM PROTEOMICS – A REVIEW

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Bovine plasma proteomics can revolutionise the field of disease diagnostics, physiological and therapeutic monitoring provided their challenges are satisfactorily addressed. It is faced by the common tribulation of the presence of large dynamic range of proteins which makes the analysis very challenging with the available conventional approaches. In the present scenario, complex multistep fractionation strategies is the method of choice for addressing the analytical challenges of the large dynamic range of plasma proteins, allowing detection of substantial number of medium to low abundance proteins with depletion of high abundant proteins (HAPs) or enrichment of low abundant proteins (LAPs) being the first pre fractionation step of these multi fractionation strategy. In fact there has been flurry of major advances in recent years offering handful of technologies for depleting HAPs or enriching LAPs offering efficacy for human plasma samples, but with few available methods for bovine. Keeping in mind the importance of livestock, this pilot review seeks to summarize the two extensively used strategies : ProteoMiner Protein enrichment technology and Multiple Affinity removal System (MARS) in relation to their successfully exploration with the bovine plasma samples.

Key words: ProteoMiner, Multiple Affinity removal System, Bovine, Plasma

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Bovine constitute an important group in livestock due to their economical values and the plasma/serum proteomics can revolutionise the field of disease diagnostics, physiological and therapeutic monitoring, but surprisingly very little work has been carried out in veterinary science. The choice between plasma and serum samples has been amply documented in the literature (Rai *et al*., 2005 and Omenn *et al*., 2005). The Human Proteome Organization (HUPO) Committee with its research collaborators concluded with the commendation that plasma is the preferred sample taken from the blood. The biological fluid, blood plasma is considered as a good representative of physiological and pathological condition of an individual's well-being due to its great intimacy with the whole body (Adkins *et al*., 2002), it contain surfeit of proteins having not only plasma-based functionalities but also possibly every other form of low concentrated proteins, it is easily accessible and noninvasive, all these characteristics render it a transcendent source for biomarker discovery. The presence of a large number of proteins in blood plasma enhances the chances of detecting proteins of significant value pertaining to a particular state/condition. On the other side of the coin it poses tremendous analytical challenge. Albumin, the most abundant protein, constitutes over half of the plasma proteins. In contrast, most of the protein, which could be probable potential biomarkers, are present in very low copy number and tends to be shrouded under the mist of high abundant proteins (HAPs), and the currently

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available technologies have limitation to resolves these low abundant proteins (LAPs) distinctly from the HAPs. So, it is in general consensus that multidimensional fractionation approach could allow detection of the medium to low abundant proteins thereby digging deep into the serum proteome and the initial step of these etiquettes is generally the depletion of HAPs or enrichment of (LAPs) as suggested by many research fraternities. In fact there has been flurry of major advancement in developing various depletion strategies preventing HAPs impeding the low abundant proteins (LAPs) and recent years has hallowed many literature citing the importance of their depletion strategy allowing detection of various signatures proteins.

Thus, depletion of high-abundant proteins or enrichment of low abundant proteins as the first pre fractionation step is becoming a routine prerequisite technique in humans for the detection and characterization of LAPs, but still in preliminary phases of in bovine. As to our knowledge limited numbers of works has been reported for preventing the impeding effects of the HAPs in bovine. Bovine plasma proteomics challenges are still in the phase to be answered, but due to the tremendous demand for urgent applications of proteomics for the betterment and management of bovine, exploitation of the pre-established method for bovine could be one of the strategies to meet urgent demand. To date, as to the best of our knowledge, the literature is actually very limited comparing the efficacies of the pre-established methods in bovine. Amongst the various strategies employed for the depletion of HAPs (Multiple Affinity Removal system (MARS), Protein A/G column, Cibracon Blue, ProteoPrep20) or for enrichment of low abundant proteins (seppro Super Enrich, ProteoMiner protein enrichment approach, protein scaffold, ProSpectrum libraries) from human samples, the two mainly employed are the Multiple Affinity Removal system (MARS) depletion column (Agilent Technologies, Santa Clara, CA, USA) and ProteoMiner protein enrichment approach (Bio-Rad laboratories, Hercules, CA, USA). So, we seek to summarize the application of these strategies in bovine.

MARS (Multiple Affinity Removal System)

MARS (Multiple Affinity Removal System) are immunoaffinity depletion column, based of polyclonal antibodies being purified by stringent affinity procedure using purified human antigens. The Fc regions of these antibodies are attached to polymeric beads through protein A cross-linking. This linkage provides easy access of proteins to the affinity-binding sites. It is a system developed by Agilent Technologies (Santa Clara, California US). The MARS beads are available in both spin cartridge and high performance liquid chromatography (HPLC) column formats and there are many versions available.

Various types of MARS columns are as follows

1) MARS Human-6 Spin Cartridge and LC column: Depletes six most abundant proteins (albumin, IgG, IgA, transferrin, haptoglobin, and antitrypsin) from human plasma/serum samples.
2) MARS Human-7 Spin Cartridge and LC column: Depletes seven most abundant proteins (in addition to above mentioned 6 abundant proteins plus Fibrinogen) from human plasma/serum samples.
3) MARS Mouse-3 Spin Cartridge and LC column: Depletes three most abundant proteins (Albumin, IgG and transferrin) from mouse plasma/serum samples.
4) MARS Human-14 Spin Cartridge and LC column: Depletes fourteen most abundant proteins (apart from original six HAPs plus fibrinogen, α2-macroglobulin, α1-acid glycoprotein, IgM, Apo lipoprotein AI, Apo lipoprotein AII, complementC3, and transthyretin) from human plasma/serum samples. It is the most recent

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technology and the depletion efficiency is 95%to 99%of the 14HAP.

The efficacies of MARS columns in depleting HAPs in human plasma/serum samples are well documented (Stone et al., 2010). These strategies has also been employed with other biological fluids whereby application of this strategy has led to profiling of proteome in human plasma, CSF, and other biological fluids as per many reported (Echan *et al*., 2005, Mathivanan *et al.,* 2008, Shen *et al*., 2006, Wang *et al.,* 2005, Cho *et al.,* 2005 Whiteaker *et al.,* 2007, Brand *et al.,* 2006*).* In one of study, researcher applying 3D LC separation and MS/MS analysis, with MARS based immunodepletion as first fractionation step, allowed detection of 10 potential biomarkers to be identified from the 3000 identified proteins (Sheen *et al* ., 2006).

Considering the importance of other mammalian species Dr. William Barrett from Agilent technologies have examined the applicability (cross reactivity) of the MARS 6 column (deplete six high abundant proteins:albumin, IgG, IgA, transferrin, haptoglobin, and antitrypsin from serum/plasma samples) for the processing of serum samples from other species including Macaca Mulatta (Rhesus Monkey), Canis Familiaris (dog), Ratta Norvegicus (Rat), Mus Musculus (Mouse) and Sus Scrofa (swine) and documented that it is optimized for the depletion of Rhesus Monkey serum samples, while dog serum samples showed similar depletion of the targeted high abundant proteins with the except for transferrin, while samples from Rat, mouse and swine showed lesser depletion of the targeted proteins as detected through 1 D gel analysis.

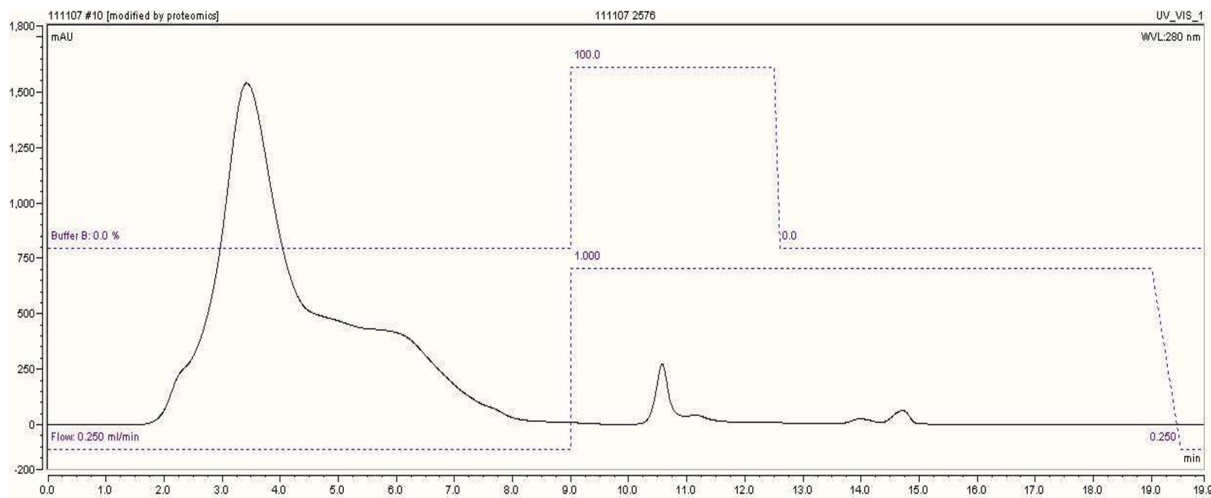

Fig1. Chromatogram of Bubalus bubalis (plasma sample on Multiple Affinity Removal System (MARS 6 column). Peak between 1.0 – 8.0 mins represent the flow through fraction while Peak between 10 - 11 mins represent the elution fraction of the bound protein. The peak for the elution fraction tends to cover more area when processing the human samples, exemplifying their efficiency in humans as cited by literature.

This study while maintaining the selectively and specificity of MARS column for the human samples as very well documented in literature, explored its applicability in monkeys and few closely related species as depicted in the table 1.

Table 1.The table depicts the Relative Total Protein Removal for Six species.

Species	% Relative protein depletion
Human	85%
Monkey	84%
Dog	81%
Swine	42%
Mouse	35%
Rat	23%

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These results are justified by the close proximity of monkeys with humans. Keeping in view the proximity of bovine with humans we have also explored the efficacy of MARS 6 column in depleting HAPs from bovine samples and found it working fairly well in depleting the targeted proteins from bovine samples (unpublished data). The LC MS/MS data revealed that amongst the six HAPs depleted through MARS column albumins and serotransferrin were detected in the study, as can be seen through the chromatogram (fig.1) the elution fraction have smaller coverage and as per literature the elution fraction peak tends to have more coverage depicting its maximally hood in humans as based on human antigen but at the same time it also demonstrate that MARS system intends to be useful for processing of bovine samples as it allowed detection of hemopexin proteins that remain shrouded under the mists of Albumin when using non-treated (un-depleted) bovine samples, though not 99% efficient in depleting HAPs from bovine, but can eliminate them to the extend preventing their masking effects, thereby exemplifying their applicability in bovine. Thus our study shows that MARS system seems to be useful for processing of bovine samples.

Limitations of MARS column approach

There are some limitations associated with this approach. Since these are based on an immunological approach, represent species dependence, thus the potential applications to samples of veterinary interest are limited. Also it works with lower amount of starting material The amount of the starting material is (8–160 µl) in humans, which is indeed a small volume and while processing the bovine samples it has to be further lower as the column is based on human antigen thereby limiting the sample volume for further analysis. Also there are some usual issues of co – depletion associated with the immunodepletion strategies, implying the depletion of other unique proteins which remain associated with the targeted HAPs.

Combinatorial hexapeptide ligand libraries (ProteoMiner protein enrichment approach)

A distinct and a ground breaking approach could be enrichment of LAPs applying combinatorial hexapeptide ligand libraries, which are commercialized under the brand name of ProteoMiner protein enrichment (Bio-Rad laboratories, Hercules, CA, USA). In doctrine, each of the unique hexapeptide bound to chromatographic beads are present in specific concentration and binds to a unique protein recognition site in the sample applied. The HAPS quickly saturate their hexapeptide ligand and excess proteins are washed out during the subsequent washing steps while low abundant species are bound completely. Thus all the proteins in the original reference sample are present but with a narrower dynamic range of protein concentrations. This technique can be used to analyze a wide array of different samples: crude extracts, purified or prefractionated samples, highly diluted protein solutions or recombinant proteins. In a nutshell HAPs saturate their ligands quickly; thereafter exceeding proteins are washed out during the procedure. In contrast, LAPs are concentrated on their specific ligands, thereby decreasing the dynamic range of proteins in the sample. The ProteoMiner technology, since it is not based on an immunological approach, should be species independent, and thus of potential application to samples of veterinary interest. Veterinary applications will thus benefit from a simple method to enrich for low-abundance proteins.

Thus as discussed above, this ProteoMiner technology is an enrichment method and not a depletion method to remove high-abundance proteins, thereby surpassing the issue of co- depletion associated with other immunodepletion strategy, thereby ProteoMiner treatment would allow detecting more proteins that escape from detection when using non-treated samples and probably other

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immunodepletion strategies. It has tasted all the hooks and corner of the proteomics samples be it urine, CSF, plant crude extract or some other sample and, the successful application of ProteoMiner approach has been demonstrated in many publications over the last five years. (Castagna *et al*., 2005, Mouton-Barbosa *et al*., 2010).

Various commercially available ProteoMiner Systems

1. ProteoMiner Small-Capacity Kit: This protocol has been optimized for plasma and serum samples with protein concentrations of greater than equal to 50 mg/ml (requires total protein load of approximately10 mg).

2. ProteoMiner Large-Capacity Kit: This protocol has been optimized for plasma and serum samples with protein concentrations of greater than50 mg/ml (requires total protein load 50 mg).

3. ProteoMiner Sequential Elution Large-Capacity Kit: This kit combines the ProteoMiner large-capacity kit and the ProteoMiner sequential elution reagents and is designed to provide multiple elution options for researchers using SELDI or other downstream protein separation analysis methods, and who wish to access additional proteins, but this is not compatible with 2-D gel electrophoresis approach.

Recently Marco-Ramella and Bassols reported the efficiency and reproducibility of the ProteoMiner protein enrichment approach with bovine and porcine samples while comparing its efficacy with the immunodepletion strategy (Marco-Ramella and Bassols, 2010) supporting their applicability for bovine samples.

Limitations of ProteoMiner approach

The major limitation is that it cannot be used with heparinized plasma samples and at times we ought to have heparinized plasma because of its minimal chelating properties and relatively low cation concentration and at that time we are left with no options.

As cited in literatures that CPLLs could also be used for differential proteome analysis. However, the obvious pitfall is that CPLLs manipulate protein abundance.so the differential expression profile which is obtained really represent the actual scenario is still a debatable issue. Many authors suggest that the proportionalities of protein abundances are maintained but how far these proportionalities are maintained is not clearly understood.

Some studies have reported that there are loss of protein after prefractionation with ProteoMiner ,thus the proteins present in the original crude extract were not detectable in CPLL treated sample (Thulasiraman *et al.,*2005 and Castagna *et al.,* 2005) In point of fact, there ought to occur various vulnerable steps where proteins could be lost during the entire etiquette. For instance, though the hexapeptide library is probably present in all permutations and combinations, there is possibility for few matching ligand to be absent. There is also a possibility that weak interactions can be broken during the washing step, and the proteins will be washed out. Furthermore, others interactions might be very strong and used elution conditions are not suitable to break protein–CPLL interaction and they may remain on the column. In summation, this “loss of protein” makes the use of CPLLs in differential proteomics problematic.

All these limitations highpoints the potential challenges of bovine serum/plasma proteomics. Unless the bovine serum/plasma proteomics challenges are satisfactorily resolved, the enormous application of the bovine plasma proteomics could not be cherished

This study shows that nevertheless the two approaches give complementary results in humans. While toddling across other species especially bovine the ProteoMiner approach as not based on immunological approach, ought to be species independent, therefore seems to have great advantages. Also obtaining much larger amount of starting material would definitely benefit

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further fractionations and analyses. It also emerges as a cheaper and technically simpler approach and surpasses the issues of co depletion. All these data synergistically demonstrate that the enrichment approach seems more suitable as the first stage of a complex multi-step fractionation practices while processing of bovine plasma samples.

CONCLUSION

This kind of strategies should widen the number of applications of plasma animal proteomics and also open doors for the exploration of newer strategies for circumventing the analytical challenges of bovine serum/plasma proteomics allowing detection of LAPs which would lend hands to researches working in the frontier of animal plasma/serum proteomics.

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