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Generation of reference serum proteome map for monitoring swine health

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Generation of reference serum proteome map of pigs can be beneficial in animal and veterinary sciences since serum contains large number of tissue specific proteins which carry valuable information about the physiological and pathological conditions of the animal. As serum is rich in high abundant proteins (HAPs), that often obscure the detection and quantification of low abundant proteins (LAPs), enrichment of later can increase the chances of their detection by proteomic studies. This can be achieved by combinatorial peptide ligand library based capture and enrichment of LAPs. The liquid chromatography coupled to mass spectrometry (LC-MS/MS) based shotgun proteomics approach has been proved as a powerful tool for accomplishing proteomic analysis in search of biomarkers (Vidova and Spacil 2017). Thus, enrichment of LAPs in serum and their subsequent analysis by LC-MS/MS is very much promising to dig deeper for detection and identification of large number of serum proteins. In the present study, a reference porcine serum proteome map was generated by analysing LAPs-enriched pig serum using nano electrospray ionization (ESI) LC-MS/MS to identify the proteins involved in different biological functions and pathways.

Four clinically healthy Large White Yorkshire (LWY) females were selected and blood samples were collected aseptically from animals by puncturing the vena cava. Sera were separated, mixed with protease inhibitor cocktail and stored at –80°C. All the animal experiments were approved by Institute Animals Ethics Committee (IAEC) of CVSc, CAU, Aizawl, Mizoram, India. The protein concentration of serum samples was estimated using automated clinical chemistry analyzer and serum samples from all the four animals were pooled by taking equal amount of protein. To enrich LAPs, ProteoMiner^{TM} kit (Bio-Rad, Hercules, CA, USA) with hexapeptide ligand library was employed according to the manufacturer's instruction. Protein sample was subjected to nano ESI-LC MS/MS analysis thrice (technical replicates) for their identification at Valerian Chem Private Limited, New Delhi, India, following the procedure mentioned in our previous study (De *et al*. 2019).

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Protein identification was carried out based on the detection of at least one peptide with a false discovery rate (FDR) less than 5%. All the identified proteins were classified based on molecular function, biological process, cellular component, protein class and pathway using PANTHER software. To understand the possible interactions between the identified proteins and visualize the protein-protein interaction (PPI) network, STRING software was used. The DAVID software was used to locate the identified proteins in classified metabolic pathways present in KEGG pathway database.

A total of 224 distinguished proteins were identified; among them, 181 proteins were identified with 1% protein and peptide FDR cut-off. Molecular mass of the majority of the identified proteins was below 60 kDa and maximum number of proteins has pI value in the range of 6 to 7. Functional classification of the identified proteins based on molecular function revealed the involvement of most of the proteins in binding (37.5%) followed by catalytic activity (32.9%). On the basis of biological process, proteins were classified into those involved in metabolic process (21.8%), biological regulation (13.9%), and so on. Classification on the basis of cellular component showed that majority of the proteins are of extracellular region (41.3%), followed by cell (22.5%). Further, classification based on protein class revealed most of the proteins belong to three major protein classes including hydrolase (22.3%), enzyme modulator (21.1%) and signaling molecule (10.8%). Pathway analysis showed the involvement of maximum number of proteins in blood coagulation (28%). PPI network analysis by STRING software revealed 3 distinguished clusters of proteins involved in blood coagulation cascade, regulation of blood coagulation and complement cascade of innate immune system. Moreover, majority of these proteins were also located in the coagulation and complement pathways present in KEGG pathway database.

So far, proteome maps of pig serum have been generated by Miller *et al*. (2009) and Zhang *et al*. (2012) but, in both the cases protein profiling was carried out without enrichment of LAPs thereby increased the chance of missing some important proteins present in very low

concentration. Therefore, in the present study, we used hexapeptide-based technology (ProteoMiner^{TM}) for enrichment of LAPs in pig serum. The ProteoMiner technology is not based on immunoaffinity depletion approach, thus species-independent and has potential application in enrichment of LAPs in serum samples of veterinary interest (Boschetti *et al.* 2019). Further, we analyzed the LAP enriched serum proteins using nano ESI LC-MS/MS which is one of the advanced MS methods for sensitive protein identification and thus increased the chances for detection of more number of proteins by overcoming the disadvantages of traditional 2-D electrophoresis and other mass spectrometric approaches (Sidoli *et al*. 2017). Some of the proteins identified in our study including complement components, coagulation factors, inter alpha trypsin inhibitors, serpins, ceruloplasmin, etc. had also been reported in pig serum proteome map earlier (Miller *et al*. 2009, Zhang *et al*. 2012). Identification of complement and coagulation cascade proteins is justified as both these systems are first line of defence against injurious stimuli and invaders and a well established cross-talk exists between these two systems. Importantly, proteins including pregnancy zone protein, fibulin-1, apolipoprotein M, pentaxin, vitronectin, thrombospondin-1, afamin, chromogranin-A etc. were not reported in earlier pig serum proteome maps. Some of these proteins have been previously reported to be potential markers of patho-physiological conditions in pig as well as in other species. Proteins including glutathione peroxidase, pregnancy zone protein, thrombospondin-1, serpins, mannose-binding lectin-C etc. have been reported to be potential biomarkers for physiological processes like pregnancy (Jalali *et al*. 2016, Kolakowska *et al*. 2017, De *et al*. 2019). Whereas, vitronectin was proposed as potential biomarker to predict feed efficiency in young pigs (Grubbs *et al*. 2016). Likewise, chromogranin-A was shown as a marker of acute stress in pigs. Proteins like afamin, fibulin-1 were reported to be involved in various disease states in human (Dieplinger and Dieplinger 2015). Involvement of maximum number of proteins in molecular functions like catalytic activity and binding and mostly of extracellular origin is in agreement with the findings of previous studies in pig, cattle and horse (Ozgo *et al*. 2015, Lepczyñski *et al*. 2018). Detection of considerable number of intracellular proteins in serum also suggests that the analysis of pig serum proteome can provide valuable information in predicting cellular dysfunction and pathophysiology of the animal.

SUMMARY

To the best of our knowledge, it is the first report on pig serum proteome map generated with LAPs enriched serum using nano ESI LC-MS/MS method. A total of 224 proteins were identified including several proteins not yet reported in earlier pig serum proteome maps. Moreover, identification of many intracellular proteins indicates their usefulness in determination of altered cell functionality. Altogether, the identified proteins in our study can serve as baseline prerequisite for future analytical studies to understand different patho-physiological conditions in pigs.

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