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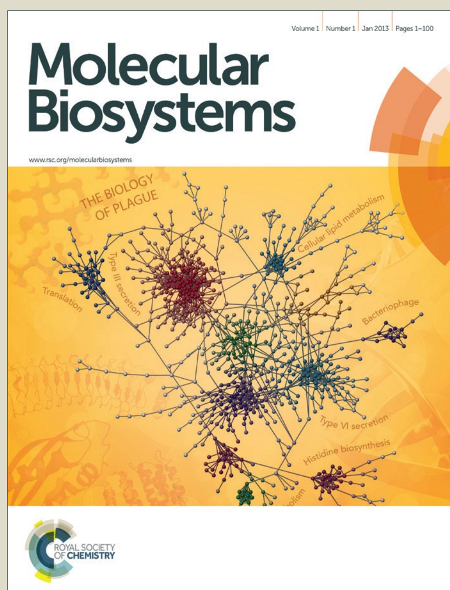

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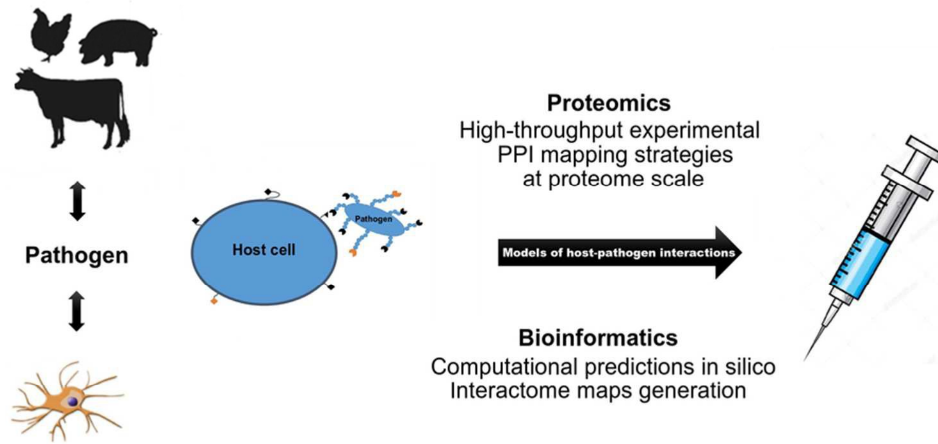

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High-throughput proteomics and fight against pathogens

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Abstract

Pathogens pose a major threat for human and animal welfare. Understanding the interspecies host-pathogen protein-protein interactions implicates novel strategies to combat infectious diseases through the rapid development of new therapeutics. The first step in understanding host-pathogen crosstalk is to identify interacting proteins in order to define crucial hot-spots in the host-pathogen interactome, as proposed pharmaceutical targets by means of high-throughput proteomic methodologies. In order to obtain holistic insight into the inter- and intra-species bimolecular interactions, upgrading the proteomic approach, sophisticated *in silico* modeling is used to correlate obtained large data sets with other omics data and clinical outcomes. Since the main focus in this area has been directed towards human medicine, it is a time to extrapolate the existing expertise to new emerging field the 'systems veterinary medicine'. Therefore, this review addresses high-throughput mass spectrometry-based technology for monitoring protein-protein interactions *in vitro* and *in vivo* and discusses pathogens cultivation, model host cells and available bioinformatic tools employed in vaccine development.

Keywords: high-throughput proteomics, host-pathogen interactions, mass spectrometry, pathogen, protein-protein interactions, quantitative proteomics, bioinformatic tools, vaccine

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Introduction

Infectious pathogens such as bacteria, fungi, protozoa, viruses and prions pose a major threat to global human and animal health, quality of life and overall economic development.[1] The majority (60 %) of infectious diseases are classified zoonosis.[2, 3] Transmission of zoonotic pathogens can occur through direct human-animal contact, arthropod vectors, aerosolized pathogens inhalation and contaminated food ingestion. Fungal diseases are transmitted mostly by direct contact and airborne pathways.[4] About 70 % of zoonoses originate from the wildlife-livestock interfaces.[3] High phylogenetic proximity of competent hosts facilitates pathogen infection and circulation, increasing pathogen prevalence. Globalization, human impact on ecosystem and constant environmental changes increase the risk of emerging new infectious diseases, the resurgence of old ones, occurrence of new drug resistant pathogen strains and their transmission dynamics. Some newly emerging infectious diseases like Zika virus, Ebola, SARS (severe acute respiratory syndrome) and H5N1 influenza (bird flu) alarm the evolution of pathogens, which may threat the human kind in near future.[4, 5] Resurgent infectious diseases are occurring due to pathogen adaptation to environment altered by humans and/or new vectors.[6] Increased vector population densities caused by climate changes and travel related exposure have enabled some of the vector borne diseases such as West Nile viral infection, malaria, leishmaniasis, trypanosomiasis, Lyme disease, dengue fever and plague to resurge causing recent epidemics in Europe, USA, India and Arabic countries, although they were thought to be effectively controlled.[7] Foodborne diseases (such as salmonellosis, toxoplasmosis, rotaviruses, listeriosis, *etc.*) are a global burden causing acute and chronic syndromes, as well as mortality.[8, 9] Rapid evolution of pathogens, especially regarding new resistance mechanisms (e.g. drug resistance in *Leishmania* and *Plasmodium* parasites, methicillin-resistant *Staphylococcus aureus*; MRSA) results in their adaptation to host environment.[10, 11] The spreading of drug-resistant pathogens

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has been accelerated due to misuse, overuse and abuse of antimicrobials causing a disability to treat common infectious diseases and a public health crisis.[11]

Three domains are necessary to combine to fight against pathogens: i) complete picture of host immune response, ii) comprehensive knowledge of the immune evasion mechanisms of the pathogens, and iii) pathogen drug resistance mechanisms. Those domains rely on the knowledge derived from the studies of intra- and interspecies protein-protein networks,[12] receptor-ligand interactions[13] and protein-nucleic acid interactions.[14] Monitoring of protein-protein interactions (PPIs) has become the current trend and major objective of systems biology.[15] It has been reported that 80 % of the proteins in the cell operate exclusively in protein complexes, whether transient or persistent ones.[16] For that reason, revealing the PPIs is unavoidable for determination of basic cell biochemistry and physiology, with the special emphasis on protein function, multi-protein complexes, as well as molecular and signal transduction pathways in both host and pathogen.[17] Interspecies PPIs reveal pathogen infection strategies and progression in aspects of pathogen ability to colonize specific niches, undermine host transcriptional and protein networks and evade immune response of the host. The main attention is focused on the specific interactions between bacterial proteins (ligands) and host receptors that are often responsible for tissue tropism and species-specificity of pathogens.[18] These interactions mediate bacterial colonization and invasion, and result in production of the factors that cause tissue damage, manipulation of cell machinery and evasion of the immune response. Treatment and prophylaxis of infectious diseases are crucial steps to improve the quality of life, avoid the economic losses and establish the health of population. Current situation calls for a comprehensive research to find new therapeutics for the cure and prophylaxis of infectious diseases. In majority of the cases, development of new therapeutics relies on PPI, mainly protein candidates crucial for pathogen survival, infection progression or host outcomes.[19]

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Historically, the scientific research in the field of infectious diseases has been focused on PPIs at the small scale; one specific pathogenic protein, mostly from the population of secreted proteins (e.g. bacterial virulence factors) and the interacting host proteins by classical biochemical approach. Immunochemical and genetic methods (ELISA, Western blot, PCR) have been employed routinely hitherto.[20] Numerous studies have provided the detailed insight into pathogen infection mechanisms, which resulted in the development of efficient drug therapies that are in current use. Nevertheless, this approach could not reveal the complexity of PPIs of species involved. In recent years, the technical and technological advances enabled comprehensive investigation of host-pathogen interactions at the molecular level using high-throughput proteomic methodology such as mass spectrometry and protein microarray technology.[21] Dealing with large-scale protein analysis, proteomics reveals protein identities, expression levels, posttranslational modifications, structural information as well as function, localization and interactions through subsequent bioinformatics analysis.[9, 22, 23] Not only the existing powerful proteomic tools (Figure 1), but also routine whole genome sequencing of pathogen species and data rich interactome analysis became the key prerequisites for investigating pathogen virulence and infection progression.

Recently, the host-pathogen interest domain has been expanded from single bacterial virulence factors to membrane proteins, surface proteome, protein posttranslational modifications, bacterial vesicles (exosomes) and related interacting host proteins. Virulence factors control and promote bacterial internalization, survival and replication in host cells. Numerous virulence factors are posttranslationally modified, especially glycosylated.[9] Differences in glycosylation are proved to be strain-dependent and have a potential role in pathogenesis and pathogen dissemination.[24, 25] Differences in surface protein and glycolipid glycosylation (e.g. in *Leishmania* or *Trypanosoma* spp.) as well as differences in biosynthetic steps of species-specific glycan moieties incorporation can be promising targets for vaccine

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development.[26, 27] Recent studies have been focused on exosomes or extracellular vesicles rather than single extracellular proteins that are released from the envelope of growing bacterial pathogens. Vesicles play important roles in establishing a colonization niche, carrying and transmitting virulence factors (proteins, lipids and nucleic acids) into host cells, and modulating host defense and response. The vesicles containing active virulence factors originating from Gram-negative pathogens such as *Borrelia* spp. have been recently reviewed.[28] Identification of interacting host proteins (direct and indirect interactions) provides the insight into the mechanism of adhesion modulation and adaptation of host cell environment for the further infection.[22]

Application of MS-based proteomic methods for PPI analysis

Utilization of mass spectrometry (MS) in the fields of biomedical sciences (including human and animal medicine) has been rising dramatically in the past decade.[29] Although the method was originally used for the analysis of chemical and physical samples, introduction of matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) into the mass spectrometry technique enabled the ionization of nonvolatile high molecular weight molecules such as lipids, nucleic acids, peptides and proteins into the mass spectrometer paving the way for the MS-related omics technologies (glycomics, lipidomics, metabolomics, peptidomics, proteomics, *etc*.).[30] Technological advances in MS instrumentation, analytical methodology and bioinformatic tools as well as the increasing number of pathogen strains and host genome sequences enabled the use of MS-based proteomic methods for PPI studies. Mass spectrometry provides the study of extreme large proteins by employing bottom-up proteomic approach and requires very low sample amounts (especially if nano-ESI source is used).[23] Unlike identification of binary protein interactions using two hybrid systems, split ubiquitin or protein arrays, complex protein interactions can be detected using workflow that incorporates affinity (*e.g.* after chemical modification of proteins), immunoaffinity

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chromatographic separation or protein correlation profiling followed by MALDI or ESI-MS methodologies for protein identification.[31] Although there are routinely performed gel-based proteomic experiments (mostly related to pull-down assays/co-immunoprecipitation or tandem affinity chromatography),[32] on-line and off-line hyphenation of MS with liquid chromatography (LC) enabled systematic large-scale high-throughput screening methods for the identification of PPIs between the components of the bacterial and host extracellular proteome. Shotgun proteomic approach, including multidimensional protein identification technology (MudPIT), is a gel-free proteomic approach that couples different types of on-line chromatographic separation techniques (mostly strong cation exchange/reverse phase (SCX/RP) two-dimensional liquid chromatography). MudPIT enables large-scale routine analysis of complex protein/peptide mixtures and protein complexes using bottom-up approach circumventing well-known LC-MS limitations such as usage of nonvolatile salts and detergents, low protein concentration, excessive sample prefractionation steps or protein properties such as molecular weight, hydrophobicity, *etc*.[23] It can be used for both, discovery and targeted analyses, cataloguing pathogen proteins. MudPIT was successfully employed in the analysis of the proteome of *Toxoplasma gondii* for creating data repositories,[33] as well as for identification of surface-exposed outer membrane proteins of *Helicobacter pylori* and their localization.[34] The same methodology was used for determination of protein content of virulent and avirulent strains of *Haemophilus parasuis* outer membrane vesicles,[35] and for revealing the lipid raft proteome of *Borrelia burgdorferi*.[36] The main disadvantage of the MudPIT is inability to identify low abundant proteins without previous enrichment and generation of large data that are laborious to process and interpret (especially protein interspecies interaction).

Affinity MS approach includes *in vitro* or *in vivo* introduction of different tags (His_6, green fluorescent protein (GFP), glutathione S-transferase (GST), *etc*.) on C- or N-terminus of

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proteins. To facilitate purification, pathogen proteins are often *in vitro* chemically modified using specific reagents (e.g. biotinylation) enabling further affinity purification of both pathogen proteins and host-pathogen interacting proteins in one or two steps, subsequently.[34] The main disadvantages of affinity labeling are that chemical modifications or the size of the affinity tag can modify the active site providing false negative result. Tandem affinity purification followed by MS (TAP-MS) is commonly used technique for identification/confirmation of interacting host proteins in the complex with pathogen originating proteins. Pathogen fusion proteins containing two contiguous epitope tags for two-stage purification (e.g. protein A and calmodulin binding peptide linked by tobacco etch virus (TEV) protease cleavage site) usually serve as bait. After affinity purification, due to sample complexity, protein complexes are finally resolved by one-dimensional SDS-PAGE and analyzed by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI TOF-MS/MS) or ESI-LC-MS/MS according to bottom-up proteomic strategy.[37] TAP-MS methodology involving Strep-II and haemagglutinin-affinity reagents with subsequent network and bioinformatic analyses has been used for identification of human cellular host proteins targeted by 70 viral open reading frames with immune-modulating functions from 30 viral species providing a valuable information for broad and specific antiviral therapies.[38] Similarly, applying *in vitro* assays, His_6-tag and a 15 amino acid residue BirA biotinylation site (Bio) was introduced to produce recombinant *Legionella pneumophila* His_6-Bio-tagged SidM effectors to monitor host interacting Rab GTP*ase*s by TAP-MS approach.[39] Furthermore, N-terminal calmodulin tag-based TAP system and full-length *Anaplasma phagocytophilum* AptA virulence protein served as a bait for purification of interacting mammalian host proteins. According to this study, it has been shown that *A. phagocytophilum* infection reorganizes host filament protein vimentin around bacterial inclusion enabling bacterial survival.[40] Since TAP-MS technology cannot distinguish whether

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PPIs are direct or indirect, some new affinity-based approaches for deciphering host-pathogen interactions have been developed. Binary interactions between influenza A virus polymerase and host proteins during viral infection has been detected using trans-complementation of Gluc1 and Gluc2 fragments of *Gaussia princeps* luciferase. The approach is based on engineering of recombinant influenza virus Gluc1-tagged polymerase expressing a Gluc2-tagged cellular proteins involved in nucleocytoplasmic trafficking pathways after viral infection.[41] Pull-down assay is also one of *in vitro* affinity-based techniques used for determination of physical interactions between affinity-tagged protein (e.g. GST, His_6, FLAG, MYC or haemagglutinin) and unknown targeted proteins applying specific antibody immobilized on agarose or magnetic beads. Protein complexes after elution can be resolved using gel-based proteomic approach or shotgun proteomics for identifying the interacting proteins, such as virulence factors of *Acinetobacter baumannii* interacting with desmosomes, that mediate host cell adhesion.[32]

Although, the utilization of MS for monitoring of PPIs was limited to protein complexes persistent after protein extraction from living cells and subsequent purification, cross-linking converts weak and/or non-covalent interactions among proteins or their proximity into covalent bonds enabling enhanced PPI study. Cross-linking agents define the proximity of amino acid residues linked. In recent years, increasing number of PPI experiments using chemical cross-linking MS has been reported. Chemical linkers not only stabilize PPIs, but due to their finite length enable structural information of proteins in complexes together with protein identification. Cross-linking reaction can be carried out in cell lysates and living cells revealing the protein interaction networks of host-host, pathogen-pathogen, and host-pathogen PPIs and can be combined with other affinity-based protein modifications.[32] Chemical cross-linking MS was used for the large-scale interspecies study of PPIs in human lung epithelial cells infected with *Acinetobacter baumannii*. Interacting

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proteins were cross-linked by biotin-aspartate proline-PIR n-hydroxyphthalimide (BDP-NHP) which also enables affinity enrichment of cross-linked proteins/peptides. As a result, 46 host-pathogen PPIs were identified, several of which include the key *A. baumannii* virulence factor OmpA and host proteins involved in desmosomes.[32] Formaldehyde cross-linking of *Legionella pneumophila* SidM and LidA effectors and their targeted host proteins during infection stabilized protein complexes and enabled the identification of interacting proteins.[39] Similarly, reactive chemical probe-based proteomic approach involving labeling with fluorescein (Flu)/tetramethylrhodamine (Rho) dyes having N-hydroxysuccinimide (NHS) reactive group enables not only the visualization of labeled proteins in host cells, but also identification of protein effectors involved in pathogen infection process (*e.g.* protein Phe A in macrophages).[42]

High resolution mass spectrometers have enabled proteins in complexes to be identified and quantified in a single LC-MS run employing quantitative shotgun proteomics.[43] There are several most commonly applied approaches to determine relative quantification of interspecies proteins in complex: labeling approach by stable isotope labeling (metabolic labeling; stable isotope labeling by amino acids in cell culture (SILAC), or isobaric chemical labeling by isotope-coded affinity tag (ICAT), isobaric tag for relative and absolute quantification (iTRAQ) or tandem mass tag (TMT)) or label-free approach.[44] Metabolic labeling was employed in the study of proteome changes in macrophages in response to exposure to *Mycobacterium tuberculosis* lipids, in parallel with chemical isobaric tagging iTRAQ, followed by two-dimensional LC-MS/MS analyses. Comparing the obtained results, authors reported that SILAC revealed a larger population of significant changes in protein expression, while iTRAQ labeling was more sensitive for identifying minor changes in protein expression level.[45] The beauty of the chemical labeling by iTRAQ is that this labeling method allow relative and/or absolute quantification of proteins using isobaric tags enabling

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multiplex experiments (up to 8 different tags in one experiment). iTRAQ labeling was employed in porcine reproductive and respiratory syndrome (PRRS) study for identification and quantification of differentially expressed proteins in virus (PRRSV) infected pulmonary alveolar macrophages (PAMs) to underlay host response to PRRSV, proteins involved in virus replication mechanism and pathogenesis.[44] TMT tagging enables multiplexing (up to 10 simultaneous experiments). TMT tagging combined with SILAC experiments has been successfully applied for monitoring of host plasma membrane proteins deregulation upon HIV-1 virus infection.[46] Label-free quantification is based on direct comparison of identical peptides across different LC-MS/MS experiments that requires high reproducibility in sample preparation and LC sample runs as well as mass spectrometry instrumentation with high resolution and scanning rates.[47] Label-free proteomic approach has been employed for identification and quantification of dendritic cell receptors for Sendai and flu viruses.[48] Furthermore, LC-MS/MS label-free semi-quantitative approach in monitoring was used to study bovine milk proteins deregulation as host response to *Escherichia coli* infection causing coliform mastitis. Apart from high abundant proteins, lower abundance antimicrobial proteins cathelicidin-1 and peptidoglycan recognition receptor protein before and after infection with *E. coli* were evaluated.[49]

Historically, most MS (including MS/MS) analyses have included data-dependent acquisition (DDA), especially in targeted proteomic approach. Accordingly, specific ions have been used for subsequent fragmentation based mostly on their intensities in MS spectrum. As a gold standard, selected reaction monitoring mass spectrometry (SRM-MS) (or MRM-multiple reaction monitoring) is the method applied for peptide/protein quantification. A proteome wide SRM-MS assay was used to deciphering the *Streptococcus pyogenes* proteome.[50] Multiple reaction monitoring mass spectrometry (MRM-MS) has been employed for host factors quantification altered by *Streptococcus pyogenes* infection that could serve as

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novel drug candidates.[51] Introduction of orbitrap technology enabled all ion fragmentation using data- independent acquisition (DIA) strategy that systematically scans and fragments precursor ions of whole peptide population in extract throughout LC time range. DIA approach creates rich datasets making quantitative analysis more consistent and applicable to wide range of peptides/proteins selected as targeted even after bioinformatic analysis (compared to gold standard targeted approach where targeted peptide ions should be set prior to MS analysis). SWATH-MS (sequential windowed data-independent acquisition of the total high-resolution mass spectra), as DIA approach, with subsequent bioinformatic analysis enables simultaneous protein identification and quantification in both label- and label free-approach.[51] SWATH-MS methodology has been used to identify and quantify nucleic acid binding and regulatory proteins involved in the host response to HIV-1 infection of monocyte-derived macrophages some of which could serve as antiretroviral drug candidates.[52] Combining 4-plex iTRAQ labeling with subsequent SWATH-MS acquisition, proteomic profiles of *Aeromonas hydrophila*, one of the most common aquatic pathogens that causes significant harm to cultured fishes, exposed to oxytetracycline stress were obtained. Validation of deregulated proteins (upregulated translation process and down-regulated energy generation process related proteins) was performed by scheduled high-resolution multiple reaction monitoring ($sMRM^{HR}$ MS) with normalization by synthetic peptides.[53]

Host cells and high-throughput approaches

In the post genomic era, there is an increasing number of model host cell lines (derived from humans or animals) grown under controlled conditions to investigate host-pathogen protein interaction networks *in vitro* at the protein level. Expressing or knock-out model cell lines are chosen to explain host pathogenesis, influence on cellular machinery, pathogen invasion, its life cycle in host and replication.[54, 55] While 2-D monolayer cells are commonly used in host-pathogen PPI studies, 3-D multilayer co-cultured cell cultures, such as microvascular

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endothelial cells co-cultured with mixed glial cells (*in vitro* model for brain blood barrier, BBB) have also been introduced.[56, 57] The origin of model cells used in research depends on the pathogen species and host pathogenesis (Table 1). Using qualitative and quantitative MS-based proteomic methodology human epithelial cells,[58] endothelial cells,[59] human foreskin fibroblasts (HFFs),[60] hepatoma/primary hepatocytes,[61, 54, 62] human lung carcinoma cell line,[63, 64] human peripheral blood mononuclear cells,[65] mouse fibroblasts,[66] *Ixodes scapularis*-derived cell lines,[67] and inflammatory peritoneal CBA murine macrophages[68] have been chosen as host model cells for monitoring the PPIs. Expressing cell lines such as Chinese hamster ovary (CHO) or human cell lines MDCK cells are used in biopharmaceutical industry for recombinant proteins production.[69, 63] To exclude the influence of differences in protein expression changes (as potential host cell protein impurities) on the suitability of multi-product immunoassays, independently generated cell lines are monitored.[70] Proteomic profiling of cell lines grown under different conditions have been investigated to monitor the influence of cultivation parameters on proteome or PTMs changes.[71, 72]

Although the application of cell culture model compared to *in vivo* models minimizes the biological variability in experiments, the influence on just one cell line is monitored. Differences in protein expression profiles due to growth conditions should be taken into account, especially while performing quantitative proteomic PPI experiments.

Pathogens and high-throughput techniques

Pathogen cultivation is inevitable for both, diagnostics and research purposes. Pathogens having different forms can be cultivated, isolated and analyzed using omics approach in different life stages.[73] For example, proteomic analysis on protein complements originating from different life cycle stages in *Plasmodium* spp. isolated from sporozoites (the infectious form in mosquito), merozoites (the invasive stage of the erythrocytes), trophozoites (form when multiplying in erythrocytes) and gametocytes (sexual stage) was analyzed by MudPIT-

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MS. It has been shown that proteins were differentially expressed depending on the physiology of each stage. MS analysis also revealed the difference in expression of protein coded by *var* and *rif* genes involved in immune evasion only in the blood stage, which were largely expressed in sporozoites.[74] Gel-based proteomic approach was used to identify proteins from *Leishmania tropica* promastigotes involved in the process of viscerotropism of parasite isolated from infected dogs. Apart from deregulation of proteins involved in energy metabolism, protein synthesis and cell signaling, down-regulation of virulence factors such as elf1-alpha in visceral isolates, as well as enhanced expression of co-chaperon, tryparedoxin and ubiquitin, show the complexity of parasite mechanisms for survival and multiplication to establish viscerotropic leishmaniasis.[75]

With high-throughput technology, proteomic analyses of cultivated pathogens can be extended to entire proteome research covering cytosolic proteins, cell wall or surface-associated proteins, extracellular proteins (virulence factors or extracellular vesicles) and PTMs or be focused only to one of mentioned subproteomic fractions.[76] Cytosolic proteins are mostly in focus for monitoring the changes in metabolism, cell division, motility/energy transfer, transcriptional and translational processes. Expression of such proteins is differential in pathogenic compared to non-pathogenic species, like in the coccoid and infectious spiral morphology of *Helicobacter pylori*.[77] Since cell wall/surface and extracellular proteins are in direct interaction with host proteins and provide information about epitopes exposed to surface, proteomics covers their labeling, extraction, enrichment strategies, and subsequent qualitative or quantitative LC-based proteomic approach. There are different strategies to analyze cell wall/surface proteins: protein shaving by trypsin or protein labeling. Protein shaving enables identification of highly hydrophobic membrane proteins (low abundant, high pI or high molecular weight). Since trypsin is unable to penetrate the bacterial cell wall, surface-exposed localization of protein is obvious.[78] As surface-exposed trypsin digestion

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buffers, PBS (pH 7.4, with/without 40 % sucrose) or 10 mM HEPES (pH 7.4) are used for protein shaving. Further, 2D-LC-MS/MS method (SCX/RP) is used for peptide separation and protein identification.[79, 78] The main disadvantage of cell shaving is cell lysis occurring due to protease treatment and centrifugation (in order to remove cells resulting in contamination with cellular proteins or DNA).[79] This can be circumvented by changes in sample preparation procedure (milder conditions during enzyme treatment, differential centrifugation or protein labeling).[80, 78] Biotinylation (by sulfo-NHS-SS-biotin) chemically modifies Lys residues located on external domains. Biotinylated proteins are subsequently purified using magnetic beads streptavidin affinity chromatography.[79] To provide a comprehensive analysis of the biotinylated protein contents, isolated fragments can be separated electrophoretically (like *Rickettsia* cell surface proteins[81]) or employing MudPIT (8 and/or 11 step MudPIT as in *Helicobacter pylori*[34] studies) followed by subsequent MS analysis. The limitation of the strategy is a low yield and its applicability is limited to pathogens that can grow in synthetic or protein free media. In addition to protein identification, understanding host-pathogen interactions requires precise quantitative measurements of pathogen proteins. Shubert *et al.* successfully quantified 97% of the 4,012 annotated Mtb proteins of *Mycobacterium tuberculosis* by the selected reaction monitoring (SRM).[82]

In addition, proteomic methods are also employed for monitoring nucleic acids-protein interactions. Specific protein and its gene expression, as potential drug targets involved in parasite differentiation, apoptosis and host-immune response, such as *Leishmania* HSP70 protein, are of special interest. Gene interacting proteins are isolated by pull-down assay using HSP70 gene (biotinylated mRNA bound on magnetic beads) and a total parasite lysate. Captured proteins were purified and identified using gel-based proteomic methodology.[83]

High-throughput technologies for monitoring the *in vitro* vs *in vivo* host-pathogen interactions

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Host-pathogen interplay can be directly monitored through changes of protein repertoire when exposed to the host/pathogen environment. As previously reported, several strategies have been applied for monitoring PPIs *in vitro*; host cells exposed to isolated bacterial proteins or infected with pathogen.[84, 85] Not only whole subset of interacting proteins can be focused, but also changes in cellular proteome expression can be studied that is structurally, functionally and dynamically related to pathogen invasion.[86] Thanks to highly sensitive technologies, pathogen proteins in host can be detected despite their low abundances. However, it is important to note that proteomic analysis of bacterial proteins upon interactions with host cells requires effective isolation of bacterial pathogens from host cells.

Monitoring the PPIs in subcellular organelles, for example PPIs in phagosomes, are crucial to understand the host-pathogen interaction.[87] Phagosome proteome serves as a valuable readout to evaluate targeted molecules for therapeutics against intraphagosomal bacteria. Li *et al*. employed label-free quantitative proteomics with subsequent bioinformatics analysis to decipher the regulation of macrophages maturation and antigen presentation pathways in macrophages and dendritic cell infected with mycobacteria showing the promise in discovering new antigen presentation mechanisms that a professional antigen presentation cell might use to overcome the mycobacterial blockade of conventional antigen presentation pathways.[87] Other aspect of subproteome level of monitoring PPIs covers phospho- or glycoproteomes in response to pathogen or pathogen-associated stimuli. Protein phosphorylation plays a critical role in the control of protein biological activity. Recent proteomic studies of macrophages infected with *Francisella tularensis* detected differential phosphorylation of tristetraprolin, a key component of mRNA-degrading machinery that controls the expression of many cytokines (such as IL-1beta and TNF-alpha), revealing the post-transcriptional mechanism that prevents the expression of host immune response factors that control infection by *Franciscella*.[85]

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To minimize the environmental factors affecting the host-pathogen interactions, most of the experiments reporting PPIs involve *in vitro* models of infection at the level of transcriptional change and proteomic profiling. Hitherto, scanty information is available on naturally occurring host-pathogen interactions or from *in vivo* experiments. In one of the *in vivo* studies membrane shaving was applied to *Corynebacterium pseudotuberculosis* harvested from infected sheep lymph nodes in order to identify surface protein repertoire. Thirteen of thirty-one identified proteins *in vivo* originating from pathogen were not identified *in vitro*. The study also revealed host proteins present on bacterial surface, mostly antimicrobial peptides and proteins related to immune mediation.[88] High-throughput proteomic analysis becomes more challenging if pathogen resides in the tissues, due to low abundance of pathogen proteins in relation to host proteome. *Mycobacterium tuberculosis* proteome from infected guinea pig lungs (thirty and ninety days after aerosol challenge) was analyzed by shotgun proteomics. According to gene ontology analysis, cell wall, metabolism and respiration related proteins were detected and intermediary metabolism and respiration were the two major functional classes of proteins represented in the infected lung.[89] Regulation of host endothelial cell function altered by *Schistosoma bovis* in terms of modulation of host immune and hemostatic response has been monitored using mouse as a model. *In vivo* biotinylation of endothelial surface proteome of infected mouse portal vain enabled affinity purification for subsequent protein identification and relative quantification by LC-MS/MS. Among the assigned biological functions, cell adhesion molecules were found to be the most abundant.[90]

The main advantage of *in vivo* model studied with high-throughput technologies is the unfolding of complete picture of regulation of protein expression by tissue microenvironment which can be only partially achieved in *in vitro* environment.[90] Although *in vivo* experiments provide more realistic insight in host-pathogen PPI interplay, some technical difficulties

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during pathogen isolation/sample preparation are present; such as remaining of host cells and cellular debris, cell lysis due to filtration or shaving. For that reason, appropriate control samples and bioinformatic tools after data processing should be used.

Bioinformatic platforms for PPI data analysis and data integration

A plethora of available commercial and free-to-use academic software platforms[23] (e.g. Proteome Discoverer, GPS Explorer, Protein Pilot, SEQUEST, MaxQuant, Skyline, Protein Pilot, Spectronaut, etc.) enable high-throughput proteomic raw data processing to obtain protein identities and quantitative information of proteins involved in host-pathogen interplay. After obtaining such information, the next step is creation of interaction network. Not only the lack of data for interspecies PPIs, but the quality of proteomic data due to low abundance of targeted proteins, inadequate sample preparation or processing can result in false positive or negative results.[91] Having that in mind, to obtain biologically relevant information it is important to integrate bimolecular interactions at various omics levels (*e.g.* transcriptomic, genomic, proteomic, lipidomic). Deciphering of host-pathogen interactions can be complex due to the lack of appropriate animal models, incomplete pathogen genome sequences and complexity of host-pathogen dynamics.[92] Different *in silico* models predict protein interaction networks which are validated by data mining of existing literature as well as gene ontology parameters analysis, followed by *in* vivo experiments on animal models, and then refined. The development of computational methods for prediction of protein-protein interactions and validation of obtained experimental results have been in focus of scientific and commercial (mostly biopharmaceutical) community. Currently available computational approaches for predicting PPIs are based on genomic and structural information, use of network topology, literature mining/database search and machine learning algorithms utilizing heterogeneous genomic/proteomic features.[93, 94]

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Computational platforms are developed to integrate, analyze, visualize and model complex biological interaction networks regardless of system of molecular components and interactions. [91] BiologicalNetwork is an example of such platform, which enables mapping and analysis of high-throughput expression data in order to create regulatory, metabolic and cellular networks.[95] Cytoscape, an another tool for integrating bimolecular interaction networks,[96] is also used commonly for visualization of interactions (e.g. between DENV proteins and human and insect hosts proteins based on protein 3-D structural similarity between pathogen and host proteins).[92] Similar platforms, VisANT (interactive visual mining of biological interaction data sets)[97] and Osprey (visualization and manipulation of complex interaction networks),[98] are also popular among researchers using omic approach.

As a result of constant production of huge PPI related data and their processing, the number of repositories for studying interspecies PPIs available online are constantly growing.[99] Most of databases are independent or contain redundant overlapping data from publications.[94] They can serve as data source and analysis (containing both predicted and detected PPIs gene ontology, protein complex data, disease outbreak, etc.), as well as integration resource for host-pathogen interactions data (regarding genome sequences, conserved domains, gene expression data related to host-pathogen interactions).[93] PATRIC (the Pathosystems Resource Integration Center) is a database specialized for infectious diseases and associated PPI research, integrating omics data, PPIs, protein structural data and associated metadata[100] expands the knowledge of the factors directly involved at the interface between host and pathogen. The most popular repositories (according to number of citations) such as General Repository for Interaction Database (BioGRID), Database of Interacting Proteins (DIP[TM]), Biomolecular Interaction Network Database (BIND**),** Molecular Interaction Database (MINT), Human Protein Reference Database **(**HPRD) and IntAct store both predicted and detected protein-protein interactions have been recently reviewed.[94]

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The role of high-throughput proteomics in therapeutics development

Increasing the knowledge of pathogen evasion and invasion, host response and infection mechanism leads to novel therapeutic strategies. In veterinary medicine vaccines not only prevent infectious diseases in animals improving animal welfare, but also decrease the cost of animal production.[101] Since the host genome is evolutionary more stable, the complexity of host-pathogen interplay is highly affected by diversity of pathogen strains and their adaptation to the host environment affecting the outcomes of existing therapies. [102] Immunotherapeutics can unspecifically target antigens originating from host tissues. Accordingly, antigen-based vaccines need to be designed specifically based on sequence similarity at the molecular level targeting exclusively pathogen proteins to avoid cross-reactivity. [103] Exploration of interactome in order to discover novel antibacterial drugs can propose a solution for growing challenge of drug-resistant and provide protection regardless of the infecting strain's serotype. For example, proteomic analysis of three different strains of *H. pylori* revealed low similarity profile of expressed proteins[104] pointing a complexity in choosing the target molecules for drug design.

Several research groups are reporting innovative proteomic strategies, like shotgun immuno-proteomics.[104-106] Most of the potential targeted antigens in vaccine production are being selected based on their surface localization or secretion, taking into consideration their role in the pathogenicity. For example, exploiting proteomes of serogroup B meningococci,[107] *Bacillus anthracis,*[108] *Rickettsia conorii*[109] and *R. parkeri*,[110] *Streptococcus suis,*[80] *Salmonella* spp., *Neisseria meningitides,*[105] *Leishmania* spp.[106] novel vaccine candidates were identified. The application of high-throughput proteomics in drug discovery includes identification of the novel antibacterial targets and allows the understanding of the mechanism of action of the existing drugs or new potential drugs *in vitro* at the same time. Drugs action mechanisms are

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commonly monitored through protein expression profiles of bacterial cultures treated with antibiotics compared to untreated bacteria.[111]

Conclusions

Climate changes and migrations affect ecosystems worldwide facilitating transition of infectious agents. Identification of new protein candidates involved in the immune evasion and invasion is essential for development of effective therapeutics against infectious diseases. Due to state-of-the-art instrumentation and improved methodologies (from sample preparation, peptide/protein separation, data analysis to interpretation), proteomics has become a powerful tool for determining molecular identities, localization, function and interactions providing a more detailed insight into bacterial pathogenesis and host-pathogen interaction networks. These data are a key factor in the fighting against infectious diseases in both humans and animals where new strategies in drug design are needed, since the traditional approaches may fail (due to evolution of resistant strains). Implementation of systems medicine employing high-throughput proteomics combined with other omics technologies (such as genomics, transcriptomics, bioinformatics), should enable effective drug development. Since the bottleneck in the omics approach is effective analysis of a plethora of complex data generated, a proper deciphering of the tangled host-pathogen interplay becomes more tedious. Computational biology, based on machine learning and modeling, is the only effective way to digest the tedious data to come up with selection of target molecules for drug design.

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TABLE

Table 1. Cell line models revealing host-pathogen interplay *in vitro*.

Cell line	Pathogen	Analytical approach	Reference
Human epithelial cells	Coronaviruses	SILAC-based quantitative proteomics	58
Mouse *L929* fibroblastic cell line	*Rickettsia prowazekii*	Gel-based (DIGE) MALDI-MS	66
***Ixodes scapularis* embryo-derived cell line ISE6 and IDE12**	/	Shotgun proteomics	67
Huh7 cells	Hepatitis C virus	SILAC combined with one-dimensional electrophoresis separation and mass spectrometry	62
Hepatoma cells/primary hepoatocytes	Hepatitis C virus	Quantitative proteomics	61
Hep 39, Hepswx		Gel- and LC-based proteomics	54
Murine cells/ macrophages	*Leishmania major, Leishmania amazonensis*	MudPIT MS	68
Red blood cells	*Plasmodium falciparum* two culture-adapted parasite strains (3D7 and CS2)	MS-based protein profiling	65
Human peripheral blood mononuclear cells			
Brain microvascular endothelial cells (BMEC)	*Cronobacter sakazakii*	Gel-based MALDI-MS LC-based proteomics	59
Microvessel endothelial cell co-cultured with mixed glial cultures (BBB)	*Borrelia burgdorferi*	Gel-based MALDI MS	56, 57
S9 human bronchial epithelial cells	*Staphylococcus aureus*	Pulse-chase SILAC approach	112
Human cell lines MDCK (for vaccine production) and human lung carcinoma cell line (A549)	Human influenza A virus	Quantitative and qualitative 2-D DIGE	63
MCDK and Vero		LC-MS based proteomics	64
Polarized intestinal epithelial monolayers	Enteropathogenic *Escherichia coli* (EPEC)	Quantitative profiling using isotope-coded affinity tagging (ICAT) LC-MS	113
Human foreskin fibroblasts (HFFs)	*Toxoplasma gondii*	Gel-based qualitative and quantitative proteomic study	60
Jurkat cell line (CD_4^+ T cells)	Human immunodeficiency virus type 1 (HIV-1)	2-D DIGE followed by MS analysis	114

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FIGURE LEGEND

Figure 1. MS-based proteomic methods employed in deciphering host-pathogen interactomes.

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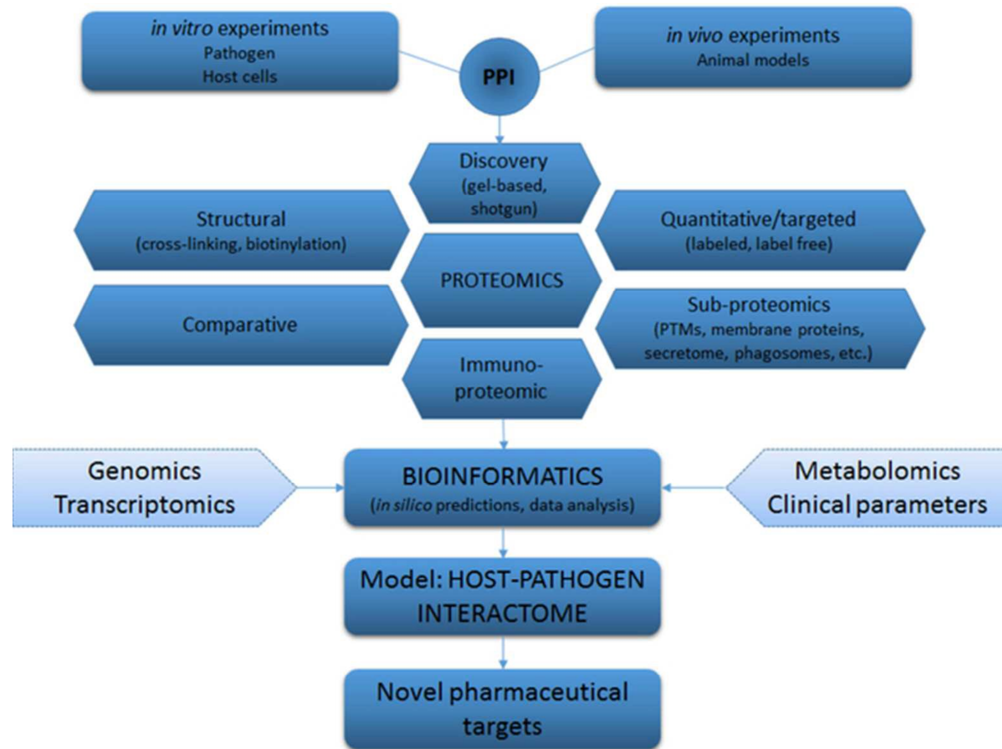

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