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Immunomic approaches for antigen discovery of human parasites

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Review

Title: Immunomic approaches for antigen discovery of human parasites

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Abstract

Introduction: Genetics combined with proteomics allows for a better understanding of parasite-host interactions and host immune responses. Immunomics elucidates that antigens are targets of induced or naturally acquired immunity (NAI), a promising solution to the challenge of eradicating human infections. High-throughput protein microarrays enhance rapid antigen discovery for the development of serodiagnostic tests/vaccines.

Areas covered: This review systematically analyzes the emergence of protein microarrays as a powerful technology for parasite antigen discovery and subsequently summarizes some of the attributes and disadvantages of these approaches. Major insights on novel/validated serological biomarkers or vaccine candidates against malaria and Neglected Tropical Diseases (NTDs) are highlighted. We conclude with a brief description of the processes involved in immunomic protein microarrays.

Expert commentary: Interesting discoveries have been made using protein microarrays. However, there is a need to evaluate targets that elicit strong immunogenicity and correlates of protective efficacy to aid prioritization and guide further clinical development. The goal of parasitic disease elimination will be best achieved through an integrated strategy that will incorporate and implement the different control components.

Key words: Immunomics, protein microarrays, human parasite, antigen, humoral response, serological biomarker, vaccine candidate.

1. Introduction

Vector-borne human parasitic diseases pose a serious threat to mankind globally. Despite concerted efforts made in the control and elimination of malaria and Neglected Tropical Diseases (NTDs), they remain major public health burdens globally, especially in the tropic and sub-tropic regions [1-3]. World Health Organization (WHO) reported 214 million new cases of malaria infection and 438,000 deaths globally in 2015 [4]. Schistosomiasis, Chagas disease, dracunculiasis, echinococcossis, food-borne trematodiases (clonorchiasis, opisthorchiasis, fascioliasis and paragonimiasis), African trypanosomiasis, leishmaniasis, lymphatic filariasis, onchocerciasis, soil-transmitted helminthiases (ascariasis, trichuriasis and hookworm disease), taeniasis/cysticercosis and trachoma are NTDs prioritized by WHO [5]. Globally, NTDs affect more than one billion people, including over 500 million children [3]. These diseases burden present serious threats to human well-being and overcoming the pathogenicity of human parasites remains a challenge due to the complexity and the variability of their functional proteome.

Novel success in global research towards elimination of parasitic diseases is someway dependent on the following; the development and validation of tools for antigen discovery, eliciting sufficiently strong immune responses to antigens, and advancing clear experimental correlates of protective efficacy to aid in prioritization and to guide pre-clinical development. Therefore, there is an urgent need to understand detailed aspects of antigen-specific human immune responses for diagnosis and vaccine purposes. Genomic sequence data and transcriptome of *Plasmodium falciparum* [6], *P. vivax* [7, 8], *Schistosoma japonicum* [9], *S. mansoni* [10], *S. haematobium* [11], *Necator americanus* [12], *Toxoplasma gondii* [13], *Trypanosoma cruzi* [14], *Echinococcus multilocularis* and *E. granulosis* [15] have boosted the post-genomic research of these human pathogens. Furthermore, the fast growth of

molecular biology has radically linked genomics to proteomics which led to post-genomic study area, termed 'immunomics' 15 years ago [16]. Previously, 'traditional' proteomics used approaches such as expression cloning, 2-dimensional (2-D) liquid chromatography elution and mass spectrometry, reverse immunogenetics to provide immune responses of target antigens [17-23]. These methods have been very useful in identifying immunoreactive antigens and also helpful in high-throughput (HTP) protein profiling. However, the combination of the so called 'traditional' methods with conventional techniques in immunology such as 2-D gel electrophoresis, western blotting and enzyme linked immunosorbent assay (ELISA) have given more insights on antigen-antibody reactivity, showing that protein targets are immunoreactive against infected individual or animal antibodies and induce immunogenicity [24].

Plasmodium malaria is the most studied human parasitic disease and there are revelations that broad antibody responses to antigens are cumulative after multiple infections or repeated exposures to the parasite, pairing with host's age, parasite prevalence [25] and immunity to clinical disease [26, 27]. Naturally acquired immunity (NAI) of host antibodies targets parasite antigens with protection against symptomatic disease [28-30] and this is mediated by both cellular and humoral immune responses [31, 32]. Furthermore, investigations in naive and semi-naive immune volunteers to evaluate the protective efficacy and attenuated vaccine studies of malaria vaccine candidates [33, 34], also, investigations to evaluate the protective efficacy of schistosomiasis vaccine candidates in healthy and resistant individuals [35, 36] have revealed consistent safety and strong immunogenicity. However, infected asymptomatic individuals remain the major bottlenecks for disease control as they influence transmission dynamics [37]. Therefore, it is pertinent to have a better knowledge of immune responses necessary to acquire clinical protection, culminating to the identification of candidate antigens.

High-throughput (HTP) protein microarrays have given the opportunity to explore and analyse both natural and experimental induced humoral immune responses of several human pathogens including: *E. multilocularis*, *E. granulosis*, *N. americanus*, *S. japonicum*, *S.*

mansoni, *T. gondii*, *T. cruzi* [12, 38-49] and the eukaryotic organisms *P. falciparum* (Pf) and *P. vivax* (Pv) [50-59]. The new concept of immunomics-based HTP assays has been set-up to change the manner in which 'traditional' immunological researches were practised.

This review highlights the emergence and effectiveness of immunomics approaches as a powerful and innovative tool for antigen discovery. Major insights on previously identified or newly discovered target antigens for the development of serodiagnostic tests and vaccines against malaria and NTDs are highlighted. Lastly, this review describes the route and workflow for this HTP protein microarray technology.

2. Immunomics protein microarrays – an innovative and promising tool for antigen discovery

Genomic research approaches have not been able to discover sufficient promising candidates for vaccine and diagnosis purposes. After the completion of genomic sequences of human pathogens [6-15], there have been revelations on their proteome. Early post-genomic research focused on HTP approaches to identify new tools for human diseases vaccine and other applications. 'Traditional' and conventional approaches have indeed enhanced the identification of target antigens that conferred evidences of significant protective immunity. *Plasmodium* malaria RTS,S vaccine and schistosomiasis Sm-p80 vaccine candidate are the most relevant examples. However, some studies based on identification approaches such as (a) expression cloning [17]; (b) naturally processed MHC-bound peptides by elution and mass spectrometry [18, 19]; (c) reverse immunogenetics for HLA-disease associations vaccine candidates [20, 21]; and (d) comprehensive screening of overlapping peptides [22, 23], could not effectively evaluate serological immune target antigens. In addition, the use of conventional techniques such as ELISA or two-dimensional protein gels to study antibody specificity and reactivity against target antigens have not been able to evaluate HTP antibody profiling [24]. These approaches seem weak considering the complexity of the immune responses, antibody profiling, and also inadequate to efficiently identify large amount of proteins. However, they are good complement to immunomics

approaches.

The ‘fabrication’ of protein chip arrays was initiated 17 years ago. The study developed a technique for high-throughput gene expression and commercial antibody screening on chip-size protein microarrays [60]. Protein chip ‘fabrication’ has been further improved over time to elucidate enzymatic analysis and proteins interaction [61, 6 2]. Interestingly, *T. gondii* human infection-based serum antibodies were the first to be targeted by microbial antigens using protein microarrays [63]. These authors validated protein microarray as a suitable assay design for the serodiagnosis of infectious diseases. The first human parasite-based HTP protein microarray technique was performed in 2006, using Pf antigens and serum from malaria-infected individuals [58]. The study provided significant understanding of humoral immune responses to *Plasmodium* by evaluating antibody–antigen binding activity. Two years later, the approach was performed with more efficiency, profiling antibody responses using Pf antigens as targets and Pf-exposed antiserum [59]. This effort culminated in protein microarrays profiling clear antibodies response and immunodominant antigens. Thus, this technology provides general information about receptor and ligand interactions.

3. Protein microarrays: advantages, disadvantages and applications

This review defines protein microarrays as the reactivity of antigens with antiserum from parasite-exposed animal or individual. Antigen peptides are fixed probes and serum antibodies are targets. In other words, the approach detects the magnitude of antibody responses to proteins regardless of their abundance, by screening the serum of pathogen-exposed individuals or animals. Highly reactive proteins are further assessed as putative vaccines, whereas antigens for diagnostic are selected by comparing different cohorts of exposed and unexposed serum samples. In addition, its unique methodology for construction, time efficient and cost-effective method, unique platform to rapidly profile variant-specific humoral responses to infections, are some of the attributes that we compared to other protein microarray techniques (Table 1).

Immunomics protein microarrays have been an excellent application to the genome of

human pathogens because it gives a better understanding of parasite-host interactions and host immune responses [12, 52, 59]. Proteome chip arrays are used to identify disease clinical protection in asymptomatic and NAI individuals [64-73]. For example, recent studies carried out in Peruvian Amazon demonstrated that Pf blood-stage proteins are highly immunodominant and clinically immune using infected asymptomatic individual sera [65, 67]. Other investigations have validated potential immune biosignatures that were previously reported for vaccine development. These include; Pf apical membrane antigen 1 (PfAMA1), merozoite surface protein 1 (PfMSP1), Pv rhoptry-associated membrane antigen (PvRAMA) merozoite surface protein 10 (PvMSP10), merozoite surface protein 1 (PvMSP1), merozoite surface proteins of Pv (Pv41, Pv12) etc. [74-82]. Such approach makes this innovative technology a powerful tool for antigen discovery/validation that can transform vaccine research and improve the development of serodiagnostic tests for human diseases. In summary, the applications of protein microarrays are: to better improve our understanding of host humoral immune responses, evaluation of protective efficacy, diseases progression through cohorts, epidemiological research, development of improved serodiagnostic tests, discovery of subunit vaccine antigen candidates and vaccine development.

However, protein microarrays are limited to humoral immune response studies due to their inability to identify non-protein antigens (polysaccharides and glycolipids). Other limitations to this technology include; the complexity to regenerate protein folding and multimerization on a microarray platform, and post-translational modifications (phosphorylation and glycoslyation) that may not be identified simply because they cannot be reproduced through *in vitro* transcription and translation reactions. Such limitations impede the discovery of new immunoreactive antigens with various post-translational modifications (Table 1).

4. Antigen discovery of human parasites using protein microarrays

Protein microarrays have emerged as a powerful tool targeting antigen discovery in the last ten years and its effectiveness is not in doubt. We have searched PubMed database with

query: ‘immunomics’ OR ‘immunoproteomics’ OR ‘protein microarrays’ OR ‘proteome array’ OR ‘serological AND humoral response’ OR ‘antibody AND biosignature’ OR ‘antigen discovery’ OR ‘blood-stage target’ OR ‘antigen candidate’ OR ‘human parasite AND vaccine candidate’ OR ‘antibody profiling’ OR ‘natural immune response’. Unrelated references and reviews or comments were excluded. The criteria for selection were based on the following; (1) research articles on human parasite/parasitic diseases and (2) antigen identification using protein microarrays. A list of forty-four articles was retained from 2006 till date (Table 2). Over sixty percent of evidences were carried out in the past three years and, were focused on *Plasmodium* spp*., Schistosoma* spp*.* and *T. gondii* antigens (Figure 1A & 1B). Protein microarrays were conducted to detect blood-stage target antigens for the development of vaccines or serodiagnostic tests against malaria and NTDs.

4.1 Malaria antigens protein microarrays: discovery and validation

Studies on *Plasmodium* have profiled antigen-specific antibody associated to anti-disease immunity in Papua New Guinea exposed-children [69]. Other investigations have identified and validated serological biosignatures using sera of individuals from endemic areas in the western Kenyan highlands and in Yunnan province, China [54, 55]. Recently, a study has revealed that natural protective antibody responses against individuals experimentally challenged with Pf sporozoites were associated to new candidate targets [64].

The emergence of high-throughput protein microarrays has contributed to the development of numerous *Plamodium* candidate antigens, as well as to the discovery of new antigens that were not identified by ‘traditional’ methods [52, 54, 70, 71, 83]. For example, Chen *et al* [52, 57] identified 169 highly immunoreactive antigens in which 12 were well-characterized Pv vaccine candidate antigens while the remaining 157 were not reported. Furthermore, Chen *et al* profiled natural antibody response to Pf antigens and identified 30 highly immunogenic merozoite antigens including 10 well-known Pf blood-stage vaccine candidates. This study was the first to report seven proteins and two hypothetical proteins to be immunogenetic [54].

More so, Pf circumsporozoite protein; PfCSP [84], Pf apical membrane antigen 1; PfAMA1 [85, 86] and Pf merozoite surface proteins 1 & 3; PfMSP1 [87]; PfMSP3 [88] to mention a few, have been immunomics-validated and are at clinical development stages. Whereas among Pv protein microarrays-validated antigens, the leading PvCSP vaccine candidate has been assessed in clinical trials [89]. Table 3 provides more insights on some of the *Plasmodium* antigens that have been identified using protein microarrays.

4.2 Schistosomiasis and other NTDs antigens protein microarrays: discovery and validation

Serodiagnostic tools and vaccines against schistosomiasis, toxoplasmosis and other NTDs are also strongly needed. This task may be achieved by identifying parasite-specific immunodominant targets for pre-clinical/clinical evaluation designs. For this purpose, immunomics-based studies have validated a panel of potential antigens and identified novel targets for schistosomiasis [45-49, 66, 68] and other NTDs [12, 38, 39, 43].

A previous study highlighted an integrated immunoproteomics approach for the analysis of *S. japonicum* tegument proteins. Thirty highly immunoreactive tegument antigens were reported for the first time in this study [49]. Some of the microarray-validated schistosome targets (Table 4) are strongly immunologically reactive, of which rSh28GST (Recombinant 28 kDa glutathione S-transferase of *S. haematobium*), Sm-TSP-2 **(***S. mansoni* tetraspanin 2) and Sj23 (*S. japonicum* associated-protein 23) antigens have progressed to clinical trials [35, 92].

Antigen discovery studies against other NTDs have not been pronounced as in schistosomiasis. However, protein microarrays have revealed antigenicity and immunogenicity to *T. gondii*, *Echinococcus* spp., *N. americanus* and *T. cruzi* antigens. For instance, significant targets have been identified to improve serological diagnosis of toxoplasmosis acute/chronic infections. These include; exon 1 of TGME49_086450 also known as GRA5 (dense granule protein 5) and exon 6 of TGME49_095700 also called

ubiquitin transferase domain-containing protein (UBA/TS-N domain-containing protein) [38]. In addition, it is important to note that the recently identified *N. americanus* hydrolase alpha/beta domain protein, UDP-glucose 4-epimerase, flavodoxin domain protein and 16 hypothetical antigens are potential candidates that might constitute the basis for sensitive and specific serodiagnostic tests against human hookworm disease [12].

5. Overview of HTP protein microarray for antigen discovery

Here, we summarize the procedure for the development of human parasites protein microarray that we developed [54, 57]. The procedure is described as follows; (1) Collection of serum samples, (2) Selection of target genes/ORFs (open reading frames), (3) Polymerase chain reaction (PCR) amplification of ORFs - Preparation of linearized vector, (4) Cloning of target sequences; (5) Protein expression and immunoreactivity detection of His-tagged protein, (6) Serum screening, and (7) Data analysis and bioinformatics approach to immunology (Figure 2).

5.1 Collection of serum samples

Positive serum samples of interest from parasite-exposed individuals/animals and serum samples from unexposed individuals/animals as control are required. The source of the antisera to profile immune responses is important as antibody profiles may give a bias understanding of the host immune responses. In the “Expert commentary” section, we described the different cohort/study designs that are currently used to identify infected symptomatic/asymptomatic individuals or animals.

5.2 Selection of target genes/ORFs

The choice of target genes involves the following criteria; high sequence homology among

parasite species, number of exon and transmembrane domain, evidence for surface antigen expression and function prediction of selected targets. The genomic sequence information of several pathogens is available on GeneDB [94] or parasite-specific database: PlasmoDB for *Plasmodium* [91], SchistoDB for *Schistosoma* [93], ToxoDB for *Toxoplasma* [95] and *N. americanus* [12]. Furthermore, it is advised that the constructs for gene expression should not contain any nucleotide sequence for signal peptide [96] and glycosylphosphatidylinositol (GPI) anchor [97].

5.3 PCR Amplification of ORFs - Preparation of linearized vector

For the proper joining of a vector and PCR amplified insert, PCR products must have linear ends with homology to that of the vector. For example, In-fusion PCR primers should be designed such that forward and reverse gene-specific primers are converted by extension at the 5' terminus. In-fusion primer design tool (http://bioinfo.clontech.com/infusion/convertPcrPrimersInit.do) can aid to convert the designed gene-specific primers.

Vector of choice (e.g. pEU-E01-His) should be linearized by double digestion with restriction enzymes, then purified.

5.4 Cloning of target sequences

In-fusion cloning is one of the high-throughput cloning methods. The system enables linearized vector and amplified insert DNA sequences to be seamlessly joined in a ligation-independent directional cloning reaction. This property of the In-fusion process has been exploited in the design of vectors for the expression of protein with precise engineered His-tags [98, 99]. Therefore, In-fusion cloning-specific kit is recommended to clone recombinant plasmids into competent cells [57].

5.5 Wheat germ cell-free (WGCF) protein expression and immunoreactivity detection

of His-tagged protein

Cell-free protein expression system can synthesize protein with high speed and accuracy [100]. The application of WGCF system-based protocol for the discovery of malaria vaccine candidates was reported in 2008 [101]. It is unique for its ability to overcome the major difficulty in the research of human pathogen infections. For instance, the system may express recombinant proteins of genes that contain very high A/T sequences and can also successfully produce large amount of human parasite proteins.

Proteins are high-throughput expressed in a cell-free *in vitro* bilayer transcription-translation system using highly purified plasmid DNA. Crude reactions containing expressed proteins will be printed directly into microarray chips without purification. Western blot analysis is required to confirm the expression level and also to detect the immunoreactivity of the proteins.

5.6 High-throughput serum screening

The process involves screening of well-type amine arrays of serum samples from parasite-exposed individuals/animals versus parasite-unexposed individuals/animals. A detailed description of the HTP serum screening method can be found as described by [54, 57].

Bound antibodies can be visualized using different types of labelled anti-human IgG, scanned in a fluorescent microarray scanner and arrays can be quantified using fixed circle method. The higher response of an arrayed protein against a serum sample will be regarded as positive if the relative ratio of signal intensity (SI) is >2.0 compared to the immune response of unexposed serum.

5.7 Data analysis and bioinformatics approach to immunology

In order to evaluate antibody profiles to parasite antigens, two-tailed unpaired method is required to analyze data. Multiple softwares (e.g. GraphPad Prism or Origin) are usually used to analyze the correlation between duplicate spots and antibody reactivity. False discovery rate needs to be corrected (e.g. Benjamini-Hochberg method, using MULTTEST

procedure of SAS/STAT software). TIGR multi-array experiment viewer (Mev) [102] is one of the softwares currently suitable to draw and analyze the heatmap of immune responses .

With regard to profiling serological responses, immunoreactive antigens are statistically compared between infection-resistant and -susceptible host groups to select vaccine targets. Whereas antigens for diagnostics might be selected by comparing different cohorts of exposed and unexposed serum samples.

6. Expert commentary

The application of protein microarrays to identify antigens for serodiagnostic tests and vaccine development is among the strategies currently in use to facilitate the elimination of human parasitic diseases. Therefore, a good understanding of human parasite proteins that are targets of either induced or NAI will provide; (a) a better knowledge of parasite-host interaction and host immune response; (b) significant insights into vaccine development tools.

The fundamental objective of HTP immunomics protein microarrays is to discover and validate potential antigen targets for serological diagnosis and vaccine development pipeline. This approach can effectively screen thousands of antigens simultaneously, identify their humoral responses and profile antibodies repertoire with meaningful statistical analysis that cannot be achieved using ‘traditional’ approaches [103, 104]. More so, it is time efficient and the technology is cost-effective for open-reading frames HTP screening that can be used to set-up a score of promising potential diagnostic/vaccine targets. Several successes have been recorded in the extensive application of protein microarrays to target malaria parasite antigens. Therefore, there is urgent need for researchers and other stakeholders to channel efforts and resources on studies that will rapidly profile variant-specific antibodies for serodiagnosis of NTDs and give more insights on the acquisition of immunity. Hence, the use of antisera to probe parasite antigens is fundamental to achieving success in this regard

and it is dependent on the nature of the cohorts i.e. we need to know the status of the individuals or animals under study, if they are well-characterized groups of naturally resistant and susceptible individuals, or animals that have been experimentally rendered resistant.

Three study designs are used to identify individuals with clinical immunity or NAI with respect to malaria cases [50]. These include; (1) Longitudinal cohort study design: natural immune responses of protection are measured in the serum of exposed individuals. Parasite proteins probed with serum antibodies correlate efficiently with clinical protection against symptomatic disease [67]. (2) Serial cross-sectional studies: individuals are observed repeatedly but less frequent than in longitudinal cohort study design. This results in more symptomatic disease cases than asymptomatic with less clinical immunity and antibodies response appears to be short-lived [71]. (3) Case-control design: this design is based on the comparison of asymptomatic versus symptomatic individuals. Host groups with blood-stage infection in the absence of clinical symptoms are thought to develop an acquired clinical immunity compared to symptomatic cases [65]. These study designs may also be valuable for the use of antiserum when probing parasite antigens of NTDs. In addition, sterile protective immunity and immune induced prophylaxis confer adequate levels of protection to human infections. They are strongly associated with a panel of potential targets [66, 70]. Although the three study designs provide significant and useful new insights on immune responses, we recommend longitudinal cohort study design on cases using HTP protein microarrays to truly identify clinically acquired immunity. This study design can identify antigen targets that elicit strong immune responses and correlates of protective efficacy.

Immunomics-based tool is promising for antigen discovery. However, both the 'traditional' and immunomics-based approaches complement each other. Although the 'traditional' methods cannot profile antibody at high-throughput level and also screen a large amount of protein, they are still effective and should be considered for identifying antigen targets. For example, *Schistosoma* Sm-TSP-2, rSh28GST and Sj23 antigens were not initially discovered using immunomics approaches, but have successfully been assessed for clinical trial [44, 92]. There are lots of promising candidate antigens against malaria and NTDs that

have been identified by either conventional or immunomics approaches. However, the challenge with some of these candidates is their inability to elicit sufficient strong immune responses to the antigens, and the lack of clear correlates of protective efficacy to aid prioritization and guide pre-clinical development.

The goal of parasitic diseases elimination is not limited to the use of serodiagnostic tools and vaccine development alone. Other measures such as vector and reservoir control, health education, information education communication (IEC), social mobilization, water, sanitation and hygiene (WASH), chemotherapy and chemoprophylaxis, surveillance and monitoring are continuously applied to effectively control these parasitic diseases. However, an integrated strategy that will incorporate and implement all the different components to control and eliminate the transmission of parasitic diseases especially in endemic areas should be greatly encouraged.

7. Five-year view

The continued transmission of parasitic diseases has remained a serious public health burden globally. Therefore, there is urgent need to prioritize and pursue vigorously the development of vaccines that will effectively control and reduce the burden of parasitic diseases. However, good progresses have been made with the use of protein microarrays to develop serodiagnostic/vaccine tools for effective prioritization and further clinical development.

The use of protein microarrays to identify potential antigen targets is not limited to malaria, but it has also been applied to target potential antigens of schistosomiasis, toxoplasmosis, Chagas disease, hookworm disease and echinococcosis. We are optimistic that the information provided in this review will further equip and strengthen the knowledge of researchers that are interested in working on NTDs, because it explicitly address the importance and effectiveness of this promising tool.

In addition, serological diagnosis candidate epitopes of *T. gondii* (GRA5, UBA/TS-N domain-contain protein), *T. cruzi* (CA-2/B13, 60 S Ribosomal protein P2 and Ribosomal

Protein L19) were identified recently using the protein microarray technique. We anticipate that new candidates from other NTDs will be discovered. Also, we look forward to the successful completion of the vaccines based on antigen/epitope of *Schistosoma* (Sj23, Calpain, Sh28GST, Sm14, Sm-TSP-2 and Sm-p80). Our expectation for the future is that researchers across the globe would engage in studies that will elucidate sufficient strong immune responses to antigens with clear correlates of protective efficacy. For instance, the profiling of natural immune responses protection to parasite antigens correlates efficiently with clinical protection against symptomatic disease.

Hence, we anticipate that in the next five years, new antigen candidates that confer prominent protective immunity against the NTDs cited in this review would have been discovered and immunomics protein microarrays will aid in prioritizing such targets for further clinical development.

8. Key issues

- In parasitic diseases endemic areas, individuals that are continuously exposed to parasitic infections develop a significant clinical protection termed naturally acquired immunity (NAI).
- Clinical protection is mediated by both innate and acquired mechanisms engaged by T-cells or B-cell receptors against the disease. This results to a prevalence level of uncomplicated and asymptomatic disease infections.
- Serum-specific antibody responses to pathogen proteins are profiled using protein microarrays, immunomics approaches to identify immunoreactive targets for the development of serodiagnostic tests and vaccines.
- Protein microarrays offer significant advantages over 'traditional'/conventional immune-proteomics methods. Protein microarrays have the ability to screen a large variation in humoral responses simultaneously. This technology is effective for measuring the breadth and magnitude of antibody responses to highly variant proteins.
- This review defines protein microarray as the reactivity of antigens fixed as probes with

serum antibodies from parasite-exposed animal/individual as targets.

- The source of the antisera to profile humoral immune responses is important for the understanding of host immune responses. Sterile immunity or immune prophylactic methods are likely to confer significant associated levels of protection in exposed individuals/animals.
- A well-type HTP protein microarray is defined as follows: ORFs are PCR amplified, In-fusion inserted into a His-tag protein expression vector for WGCF expression, proteins are then arrayed. Antigen–antibody reactivity is detected and probes are scanned to identify antibody signatures.
- Immunoreactive antigens are compared between infection-resistant and -susceptible host groups for the selection of vaccine targets, whereas antigens for diagnosis might be selected by comparing different cohorts of exposed and unexposed sera samples.
- Protein microarray is a powerful tool to strengthen our understanding on parasite-host interactions, host immune responses and also to discover or validate potential serological diagnosis/vaccine candidate epitopes.

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Declaration of Interest

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Legends for Tables and Figures

Table 1. Protein microarray systems: advantages, disadvantages and applications.

Table 2. List of evidence obtained from PubMed searches for human parasite antigen screening using HTP protein microarrays.

 Data search up to July 2016.

Table 3[#]. Highly antigenic *Plasmodium* targets discovered or validated using protein microarrays.

[#] The list of target antigens is not exhaustive

Table 4[#]. Highly antigenic schistosome targets discovered or validated using protein microarrays.

[#] The list of target antigens is not exhaustive

Figure 1A. Distribution of references searched from PubMed for human parasite antigen identification using protein microarrays.

 Data search up to July 2016.

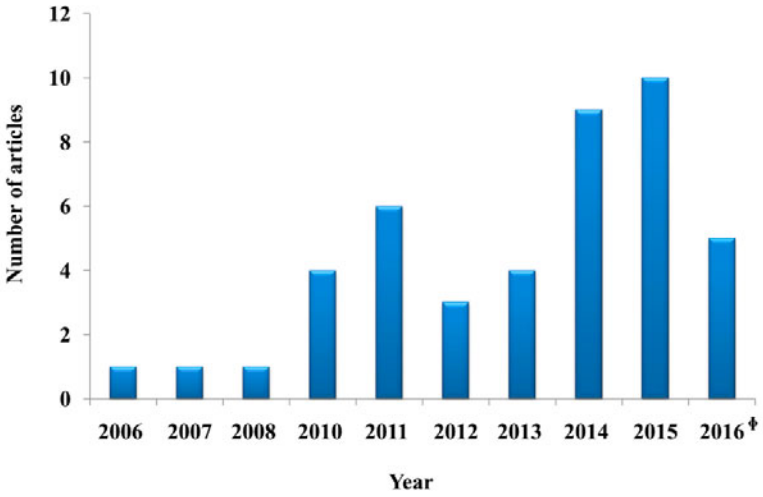


Figure 1B. Antigen screening of human parasitic infection using protein microarrays: Percentage distribution of references from 2006 till date□.

□ Data search up to July 2016.

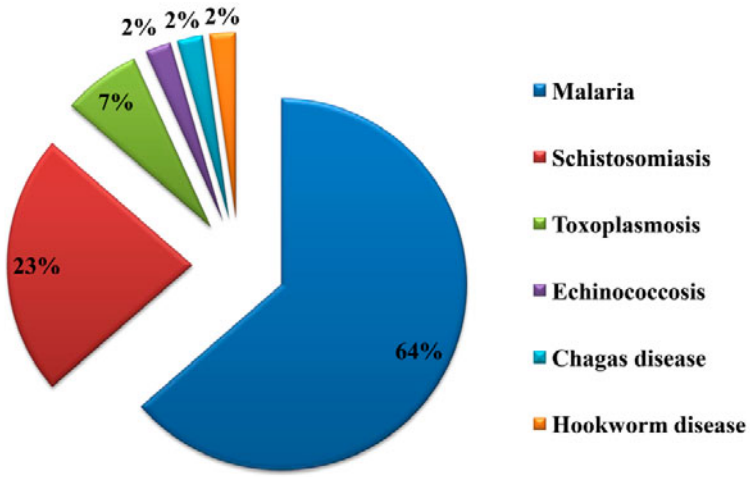
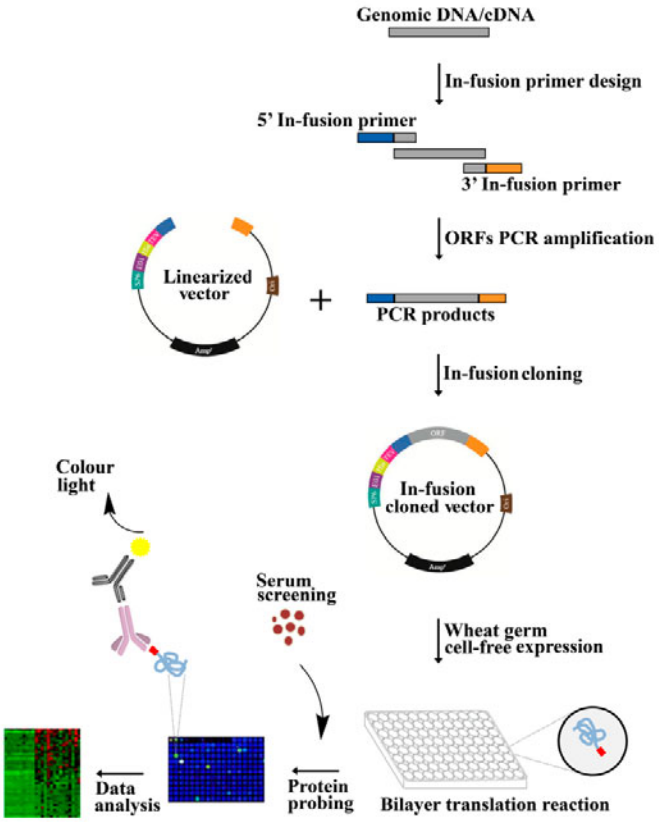


Figure 2. High-throughput protein microarrays for antigen discovery: an overview of a good workflow.



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Reference annotations
● = *of interest*
●● = *of considerable interest*

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Table 1: Microarray systems: advantages, disadvantages and applications.

Characteristics	Reverse phase protein microarrays (Target protein arrays)	Sandwich protein microarrays (Capture arrays)	DNA microarrays (DNA chip or biochip)
Principle	Antigens are fixed then probed with sera antibody.	Antibodies are fixed then probed with target proteins.	Hybridization of targets (two DNA strands) to probes.
Applications	Antibodies profiling; Identification of serological biomarkers and vaccine candidates.	Protein expression profiling in different solutions; Identification and profiling of diseased tissues.	Gene expression profiling; Identify genes whose expression is changed in response to pathogens.
Attributes	Proteome-wild; Selective expression of full-length protein; Rapid; Protein localization not affected.	Analytical protein studies; Rapid; Consuming small quantity of sample. .	Monitoring expression level of thousands of genes; Unbiased screen for relevant gene expression.
Disadvantages	Lack of protein post-translation modifications on a microarray platform.	Antibodies probed with a complex protein solution; Various detection systems.	Weak binding of complementary strands; Difficulty in interpreting results.

Table 2: List of evidences obtained from PubMed searches$^{\phi}$ for human parasites antigen screening using HTP protein microarrays.

Parasites	Related diseases	Areas covered	References
P. falciparum	Malaria (global burden)	Natural or induced antibody response profiles; Sterile protective immunity associated with blood-stage targets; Anti-disease immunity using sera from naturally infected individuals	[50, 53-56, 58, 59, 65, 67, 69-71, 73, 75, 81, 83]
P. vivax	Malaria (global burden)	Antibody profile using naïve and semi-naïve individual sera; Naturally acquired humoral response profiles. Anti-disease immunity using sera from naturally infected individuals	[50-53, 57, 64, 69, 72, 74, 76-80, 82]
S. japonicum	Schistosomiasis (NTD)	Natural or induced antibody profiles from infected hosts; Antibody signatures and disease pathologies in patients.	[2, 40, 44, 46-49]
S. mansoni	Schistosomiasis (NTD)	Antibody signatures: serodiagnostic tests and vaccine targets	[2, 44, 45]
S. haematobium	Schistosomiasis (NTD)	Sera profile of human/primates resistant to schistosomiasis	[66]
E. multilocularis	Echinococcosis (NTD)	Explorative selection of serological biomarkers	[43]
E. granulosis	Echinococcosis (NTD)	Explorative selection of serological biomarkers	[43]
T. gondii	Toxoplasmosis (NTD)	Antibody profile using different types of infected sera	[38, 41, 42]
N. americanus	Hookworm disease (NTD)	Protein microarrays profiling natural antibody responses	[12]
T. cruzi	Chagas disease (NTD)	Biomarker screenings and proteome-wide immune responses	[39]

$^{\phi}$ Data search up to July 2016.

Table 3[#]: Highly antigenic *Plasmodium* targets discovered or validated using protein microarrays.

Parasite	Gene ID	Antigen, description	Outcomes[♣] / Current status	References
Pf	PF3D7_0930300	MSP1 (Merozoite surface protein 1), GPI-AP[♣]	Validated; Vaccine candidate / Clinical trial	[50, 54-56, 59, 65, 70, 71, 75]
	PF3D7_0220000	LSA3 (Liver stage antigen 3)	Validated; Vaccine candidate / Clinical trial	[50, 55, 58, 59, 65, 67, 70, 71]
	PF3D7_1133400	AMA1 (Apical membrane antigen 1), Moving junction	Validated; Vaccine candidate / Clinical trial	[54, 56, 58, 59, 70, 75]
	PF3D7_0206800	MSP2 (Merozoite surface protein2), GPI -AP[♣]	Validated; Vaccine candidate / Clinical trial	[50, 53-55, 58, 59, 67, 70, 71]
	PF3D7_1035400	MSP3, Merozoite-associated polymorphic antigen	Novel identified and validated / Clinical trial	[54, 70, 71, 90]
	PF3D7_0731500	EBA-175 (Erythrocyte-binding antigen), EBL family	Validated; Vaccine candidate / Clinical trial	[50, 54, 55, 59, 70, 71]
	PF3D7_0304600	CSP (Circumsporozoite protein), Virulence	Validated; Vaccine candidate / Clinical trial	[56, 58, 59, 70]
	PF3D7_1335900	SSP2/TRAP[♣] , Hypothetical	Validated; Vaccine candidate / Clinical trial	[58, 59, 70]
	PF3D7_0702300	STARP (Sporozoite threonine asparagine-rich protein)	Validated; Vaccine candidate / Pre-clinical trial	[55, 56, 59, 70, 71]
	PF3D7_1036400	LSA1 (Liver stage antigen 1)	Validated; Vaccine candidate / Pre-clinical trial	[50, 55, 56, 59, 67, 70, 71]
	ID multiples[¶]	PfEMP1 (Pf Erythrocyte membrane protein 1)	Validated; Vaccine candidate / Pre-clinical trial	[55, 56, 67, 70, 71, 81]
	PF3D7_0207600	SERA5 (Serine repeat antigen 5)	Validated; Vaccine candidate / Clinical trial	[55, 70, 71]
	PF3D7_0722200	RALP1 (rhoptry-associated leucine zipper-like protein)	Novel identified rhoptry protein; Antigenicity	[54]
	PF3D7_1334800	MRSP2 (MSP7-like protein)	Novel identified and validated; Antigenicity	[54, 55]
	PF3D7_1102800	ETRAMP11 (Early transcribed membrane protein)	Novel identified; Antigenicity	[54]
	PF3D7_1401400	ETRAMP14 (Early transcribed membrane protein)	Novel identified and validated; Antigenicity	[50, 54, 55]
	PF3D7_1014100	*C*onserved *Plasmodium* protein, Hypothetical	Novel identified and validated, unknown	[50, 54-56, 59, 70, 71]
	PF3D7_0104200	*C*onserved *Plasmodium* protein, Hypothetical	Novel identified; Unknown	[54]
	PF3D7_1459900	*C*onserved *Plasmodium* protein, Hypothetical	Novel identified; Unknown	[54]
	PVX_099980	MSP1 (Merozoite surface protein 1), GPI-AP	Validated; Vaccine candidate / Pre-clinical trial	[57, 64, 77, 80]
	PVX_092275	AMA1 (Apical membrane antigen 1)	Validated; Vaccine candidate / Pre-clinical trial	[51, 57]
	PVX_119355	*Pv*CSP (Circumsporozoite protein)	Validated; Vaccine candidate / Clinical trial	[72]
	PVX_113775	Pv12, GPI-anchored protein, Cys6 family	Novel identified and validated; Antigenicity	[57, 64, 79]

Pv	PVX_097625	MSP8, GPI-anchored proteins	Validated; Antigenicity	[51, 57, 64, 72]
	PVX_114145	MSP10, GPI-anchored proteins	Validated; Antigenicity	[51, 57, 64, 76]
	ID multiples¶¶	MSP7 (Merozoite surface protein 7), Putative	Novel identified and validated; Unknown	[57, 64]
	PVX_003565	ETRAMP11 (Early transcribed membrane protein)	Novel identified and validated; Antigenicity	[52, 57]
	PVX_082680	MSP7, , Putative	Novel identified and validated; Unknown	[57, 64]
	PVX_087140	Hypothetical	Novel identified; Unknown	[57]
	PVX_081550	Hypothetical	Novel identified; Unknown	[57]
	PVX_090210	Hypothetical	Novel identified; Unknown	[57]
	PVX_094920	Hypothetical	Novel identified; Unknown	[57]

Annotations:

♣ GPI-AP: Glycosylphosphatidylinositol anchored protein

♣ SSP2/TRAP: Sporozoite surface protein 2/Thrombospondin-related anonymous protein

Gene ID annotations were selected from PlamoDB [91].

¶ PfEMP1 gene IDs: PF3D7_0223300; PF3D7_0420700; PF3D7_0800200; PF3D7_0808600; PF3D7_1300300; PF3D7_0617400; PF3D7_0800300; PF3D7_0711700; PF3D7_1100200.

¶¶ MSP7 gene IDs: PVX_082655; PVX_082680

• Outcomes:

Validated: Initially identified using conventional/'traditional' techniques and validated by protein microarrays.

Novel identified and validated: Discovered and validated using protein microarrays.

Novel identified: Newly discovered using protein microarrays.

[#] The list of target antigens is not exhaustive.

Table 4[#]: Highly antigenic schistosome targets discovered or validated using protein microarrays.

Parasite	Gene ID	Antigen/Description	Outcomes[•]/Current status	References
Sj	AY810792	Butyl-cholinesterase	Validated; Therapeutic use / Pre-clinical trial	[66]
	AY815196	Sj-TST-26 (Tetraspanin), a homolog of human TST-33	Validated; Therapeutic use / Pre-clinical trial	[44, 46, 66]
	AY815303	Microsomal glutathione-S-transferase (GST)	Validated; Therapeutic use / Pre-clinical trial	[46, 66]
	AY814430	Calpain	Validated; Therapeutic use / Pre-clinical trial	[46, 66]
	AY812195	Extracellular superoxide dismutase (Cu–Zn)	Validated; Therapeutic use / Pre-clinical trial	[46, 66]
	AAA29920	Sj23 (*S. japonicum* membrane-associated protein 23)	Validated; Vaccine candidate / Clinical trial I	[48, 68]
	ABI31730	Sj29 (*S. japonicum* membrane-associated protein 29)	Validated; Antigenicity	[40, 44, 46]
	SJC_S002499	STIP1 (homology and U-Box containing protein 1)	Novel identified; Vaccine candidate	[49]
	SJC_S013285	PPase (ADP-ribose pyrophosphatase)	Novel identified; Vaccine candidate	[49]
	AY809178	Hypothetical/ guanylate kinase associated	Novel identified; Vaccine candidate	[47]
	AY809526	SjTHBS1 (Thrombospondin 1), hypothetical	Novel identified; Vaccine candidate	[47]
	AY815838	Hypothetical Protein	Novel identified and validated; Diagnostic	[40, 47]
	AY816003	Sj-L6L-1 (Ly-6-like protein 1)	Novel identified; Vaccine candidate	[40]

	MS3_01257	Acetylcholinesterase	Validated; Therapeutic use / Pre-clinical trial	[66]
	MS3_02232	Tetraspanin-33	Validated; Therapeutic use / Pre-clinical trial	[66]
Sh	MS3_02176	rSh28GST, Glutathione S-transferase	Validated; Vaccine candidate / linical trial III	[66]
	MS3_02545	Facilitated glucose transporter member 1	Validated; Therapeutic use / Pre-clinical trial	[66]
	MS3_03655	Sodium/potassium transporting ATPase subunit	Validated; Therapeutic use / Pre-clinical trial	[66]
	MS3_02003	Calpain	Validated; Therapeutic use / Pre-clinical trial	[66]
	Smp_181530	Sm-TSP-2 (*S. mansoni* tetraspanin)	Validated; Vaccine candidate / Clinical trial I	[46, 68]
	Smp_137410	Smp80 (calpain)	Validated; Vaccine candidate / Clinical trial I	[46, 68]
	Smp_095360	Sm14, fatty acid binding protein (FABP) family	Validated; Vaccine candidate / Clinical trial I	[68]
Sm	Smp_139970	Sm-CaM-3 (*S. mansoni* calmodulin 3)	Novel identified; Vaccine candidate	[68]
	Smp_050270	Sm-CaM-2 (*S. mansoni* calmodulin 2)	Novel identified; Vaccine candidate	[68]
	Smp_194970	Sm-TSP-3 (25 kDa integral membrane protein)	Novel identified and validated; Vaccine candidate	[40, 45, 46, 68]
	Smp_161340	Sm29 (*S. mansoni* membrane-associated protein 29)	Validated; Vaccine candidate	[44-47, 68]

S. japonicum (Sj) gene ID annotations are from GeneBank (NCBI); Sh from SchistoDB [93] and *S.mansoni* (Sm) from GeneDB [94] respectively.

* Outcomes:

Validated: Initially identified using conventional/'traditional' techniques and validated by protein microarrays.

Novel identified and validated: Discovered and validated using protein microarrays.

Novel identified: Newly discovered using protein microarrays.

The list of target antigens is not exhaustive.