Next-Generation Technologies and Systems Biology for the Design of Novel Vaccines Against Apicomplexan Parasites

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Parasites of the phylum Apicomplexa are the causative agents of important diseases such as malaria, toxoplasmosis or cryptosporidiosis in humans, and babesiosis and coccidiosis in animals. Whereas the first human recombinant vaccine against malaria has been approved and recently recommended for wide administration by the WHO, most other zoonotic parasitic diseases lack of appropriate immunoprophylaxis. Sequencing technologies, bioinformatics, and statistics, have opened the “omics” era into apicomplexan parasites, which has led to the development of systems biology, a recent field that can significantly contribute to more rational design for new vaccines. The discovery of novel antigens by classical approaches is slow and limited to very few antigens identified and analyzed by each study. High throughput approaches based on the expansion of the “omics”, mainly genomics and transcriptomics have facilitated the functional annotation of the genome for many of these parasites, improving significantly the understanding of the parasite biology, interactions with the host, as well as virulence and host immune response. Developments in genetic manipulation in apicomplexan parasites have also contributed to the discovery of new potential vaccine targets. The present minireview does a comprehensive summary of advances in “omics”, CRISPR/Cas9 technologies, and in systems biology approaches applied to apicomplexan parasites of economic and zoonotic importance, highlighting their potential of the holistic view in vaccine development.

Keywords: apicomplexa, CRISPR/Cas9, omics, systems biology, vaccines

INTRODUCTION

Apicomplexans parasites are a major cause of disease in humans and animals worldwide. These pathogenic unicellular microorganisms can be zoonotic, threatening human populations, and/or compromise animal health and welfare, causing an economic impact to the farming industry. The most relevant human parasite, Plasmodium spp., is the etiological agent of malaria in humans. Other zoonotic apicomplexans are the cyst forming Toxoplasma gondii that is the model organism for research within the phylum, and Cryptosporidium parvum. Parasites of this phylum also encompass several species causing diseases with welfare and economic impact in livestock and poultry, as well as in wild animals and pets (1), some examples are babesiosis, eimeriosis (coccidiosis), neosporosis, besnoitiosis, and theileriosis, among others, caused by species from...
the genera Babesia, Eimeria, Neospora, Besnoitia, and Theileria, respectively. Although there are notable differences between apicomplexans, they are obligate intracellular microorganisms for some stages, with complex lifecycles. Typically, parasites of this phylum contain an apical complex composed of the conoid and specialized secretory organelles that includes micronemes, rhoptries, and dense granules, which are involved in host cell attachment, invasion, and the establishment of an intracellular parasitophorous vacuole within the host cell (2); proteins secreted by these organelles has been classical candidates for vaccine development (3).

The advanced molecular tools for the study of some of these organisms can provide detailed information of relevant biological processes that can be an invaluable source for antigen discovery and vaccine development. Despite that the first vaccines were developed in humans in 1796, with the smallpox vaccine (4), and no vaccines were developed in the veterinary field until a century later, being pioneer the vaccine against chicken cholera developed in 1879 (5) and a few years later against bovine babesiosis developed by Pound et al. (6), and Connaway et al. (7), which were followed by vaccines against avian coccidiosis (8) developed in the 1950s. However, the major contribution to the development of vaccines against parasites came from the efforts to combat malaria in the 1960s (9). Besides all the progress made towards the control of human malaria disease, between 2010 and 2017 the incidence has only been reduced by an 18% (10); and despite its importance in public health, there is only one vaccine approved by the European Medicines Agency against human malaria: Mosquirix (RTS,S– GlaxoSmithKline) (11, 12) that has recently been recommended by the World Health Organization (WHO) for its widespread use in children in sub-Saharan Africa and other regions with moderate to high risk of Plasmodium falciparum transmission (13). To date, only a small number of vaccines against apicomplexan parasites of veterinary importance are available (14). The main barriers in protozoal vaccinology relate to their complex and multifaceted lifecycles, presenting different stages and intricate interactions with the host, not yet well understood, impairing the development of successful vaccines.

The discovery of novel antigens by classical approaches is slow and limited to very few antigens (15). High-throughput technologies based on the expansion of the “omics” (defined as the characterization and quantification of pools of biological molecules such DNA—genomics, RNA—transcriptomics, proteins—proteomics, metabolites—metabolomics, lipids—lipidomics) has facilitated the functional annotation of the genomes for many of these parasites, significantly improving the understanding of their biology, interactions with the host (16), as well as providing novel targets for vaccine development (17). Additionally, advances in genetic manipulation of parasites have allowed the generation of transgenic populations to understand biological processes (18) or experimentally validate the gene function (19).

Systems biology is defined as an interdisciplinary approach within the area of biomedical research that combines big data derived from multi-omic studies with computational and statistical analysis, aimed to unravel interactions and dynamics from single to complex biological levels (20, 21). The use of data generated by high-throughput-omics technologies in the context of vaccination has raised the new field of “systems vaccinology”, also known as vaccinomics, aimed to understand the biological processes involved in vaccine-induced immunity as a holistic view (22). Systems biology-based approaches can improve the understanding of protective immune predicting behavior of the immune system in responses after vaccination. Given the current gaps in host-parasite interactions and the need for novel vaccine candidates, systems-biology could also help to fill-up the current empties in knowledge, allowing the discovery of potential novel targets (23) for chemoprophylaxis, vaccine development and valuable surrogate markers (24) as depicts Figure 1. The following section summarizes the advances in “omics”, CRISPR/Cas9 technologies, and the latest findings in this field of systems biology for the most relevant apicomplexan parasites.
be simultaneously processed and analyzed for both host and pathogen. Current RNA sequencing (RNA-seq), a breakthrough molecular tool, can now provide the full transcript profile (transcriptome) of cellular RNA with a large dynamic range and improved sensitivity (31). Transcriptomic studies are widely used for diverse purposes. For example, global transcriptome analysis for both host and T. gondii during the establishment of chronic infection in mice was performed by Pittman and collaborators (2014) (32). The results demonstrated the influence of parasite development on host gene transcription as well as the influence of the host environment on parasite gene transcription. Importantly, the host genes associated with the immune response were more abundant during the chronic infection than in the acute phase. Conversely, parasite genes that are highly expressed in both acute and chronic infection were involved in transcription and translation, highlighting that both stages of the parasites can actively synthesize proteins.

Comparative transcriptomics can contribute to identifying biomarkers of resistance to parasite infection, enabling a better understanding of the onset of the immune response such as was demonstrated by Bremmer and collaborators (2019) (33) in two lines of chicken with distinct resistance or susceptibility to E. maxima infection. They found that the timing at which the immune response is mounted is paramount to resistance, in particular for early induction of IFN-γ and IL-10, with a new gene identified (IL-21) associated to resistance to E. maxima infection.

Proteomics represents a step forward for the understanding of the actual proteins expressed by these genomes in specific conditions, and how well the current models can predict protein sequence. Proteomics data can supplement genome annotation efforts, by confirming gene models or correcting gene annotation errors (34). In order to improve the proteomic understanding of gene expression in these protozoa parasites, large-scale proteomic studies have been undertaken in P. falciparum (35, 36), C. parvum (37, 38), and T. gondii (39) as well as other studies of subproteomes (40, 41). The comparative study of the proteome of isolates of Neospora caninum exhibiting different virulence levels led to the identification of novel immunoprophylactic targets (42) that have been evaluated as vaccine candidates with good results in immunoprotection (43). A recent multiplexed proteomic study has demonstrated significant modulations in key physiological pathways, such as lipid metabolism, cytokine signaling, complement, and coagulation cascades in severe malaria, providing blood markers that could improve monitoring the disease progression (44).

In addition to proteomics (39), lipidomics analysis in T. gondii has been addressed in the last decade, revealing parasite-specific proteins and lipids, unraveling parasite-host interactions (45). Recently, Kadesch and collaborators (2020) (46) carried out a mass spectrometry imaging in Besnoitia besnoiti and T. gondii infection in primary bovine umbilical vein endothelial cells using atmospheric-pressure scanning microprobe matrix-assisted laser desorption/ionization (AP-SMALDI) mass spectrometry imaging (MSI), an emerging technique that provides high resolution and allows analysis of single cells, allowing a metabolomic characterization. This study has revealed biomolecular markers of infection in both parasites and has shown striking differences in the metabolites during infection between both parasites, despite their closer phylogenetic relationship, related to lipid classes such as phosphatidylcholines, phosphatidylethanolamines,
phosphatidylglycerols, cardiolipins, phosphatidic acids, and phosphatidylinositol (46).

Clustered Regularly Interspaced Short Palindromic Repeats/Associated Protein 9 (CRISPR/Cas9) Systems

There is a wide range of emerging tools adapted now to many Apicomplexa that can allow the discovery of antigens with potential interest as vaccine targets. Next-generation technologies of gene editing such as CRISPR/Cas9 have contributed to this issue, moving forward gene functional studies, by knocking-out, repressing, activating, or tagging genes in species where it was not possible before by other methods. This has enhanced and speed-up the understanding of the biology of these parasites, providing newly characterized genes as potential targets for vaccine development (Figure 1). It has revolutionized parasitology research (47), being successfully applied to P. falciparum (48, 49) in the first place, and then to other apicomplexan parasites (T. gondii, Plasmodium yoelii, C. parvum, and Eimeria spp.) (19, 50–53). For C. parvum (54) step-by-step protocols have been published, becoming an invaluable resource for the research community. Improved CRISPR/Cas9 based technologies or the generation of knock-out libraries in T. gondii have allowed the functional characterization of a greater number of genes related to virulence (55), leading to novel therapeutic targets. Recently, a CRISPR/Cas9 strategy coupled with glycomics in T. gondii has allowed new insights into the role of glycogens, and the discovery of novel genes (56). Glycans are involved in many cellular functions such as invasion, oxygen sensing, wall formation of parasite stages (cyst), and nutrient storage; hence, this study may provide knowledge regarding key cellular functions and also regarding virulence in related parasites.

SYSTEMS BIOLOGY APPLIED TO APICOMPLEXA

Systems Immunology

System biology-based approaches have been extensively applied to studies of host-parasite interactions (23) and were recently reviewed by Swann and collaborators (2015) (26). Systems biology-based approaches applied to the study, analysis, and understanding of the immune system are also known as “systems immunology”, and has been applied to identify immune signatures upon infection of Plasmodium sp. (57–60) and T. gondii (61). The analysis of transcriptomic data from both, parasite and host, has revealed some new aspects of parasite immunology and are summarized in Table 1. A recent study carried out using blood from people with uncomplicated P. falciparum malaria indicated that the innate immune response, cytokines (IL-1β, IL-6, TNF-α, and IFN-γ), and apoptosis pathways were acutely upregulated in the group under study with concomitant downregulation of immune-modulatory and apoptosis inhibitory genes (58). These results are in contrast with a previous report from Ockenhouse and collaborators (2006) (57) that showed that genes involved in phagocytosis and inflammation, including the cytokines TNF-α, IFN-γ, and IL-1β were downregulated. The results and the different interpretations that raise from the different datasets may be affected by variations in the population under study, clinical phenotype, and vaccine regimen. Likewise, due to the high degree of heterogeneity between biological samples, high variability is expected. Additionally, immune responses are affected by other factors such as age, genetics, stress, comorbidity, that hinder the interpretation of the immune mechanisms. Therefore, the more data is collected the more hypothesis would arise and novel findings and biomarkers will be generated, clarifying this issue.

Yamagishi and collaborators (2014) have performed a transcriptomic analysis both in P. falciparum and humans to elucidate the mechanism of host interactions. This work has identified human and parasite genes and pathways that correlated with various clinical data (Table 1), providing novel targets for therapy. Furthermore, it has been identified genetic variations in anti-malaria drug resistance-related genes as well as associated with severe malaria symptoms (59). Another transcriptomic analysis focused on avian malaria has revealed differences in the sets of RNA between infected and uninfected birds (Eurasian siskins), demonstrating shifts in response to malaria infection (60) (Table 1).

A recent study has shown that microRNA (miRNA) expression in pigs is altered by T. gondii. In addition, genes related to immune responses are differently regulated when compared to the splenocyte miRNA expression profiles during acute and chronic toxoplasmosis (61) (Table 1).

Systems Vaccinology

Systems biology-based approaches have been applied in malaria research in immunology and vaccine development since the last decade. Tran and Crompton (2020) (66) have reviewed and well-documented all the information regarding this issue. It is important to highlight that a well established standard protocols are necessary for the assessment of vaccine efficacy for each disease to allow comparative evaluation. Studies in malaria infections have adopted a standardized safety procedure, known as controlled human malaria infection (CHMI) that has been applied, among other purposes, to the fields of malaria vaccine and drug development, supporting immunology studies (67) and systems vaccinology approaches.

Among studies carried out on malaria in systems vaccinology, it is interesting to mention that Vahey and collaborators (2010) (62) have found genes of the immunoproteasome pathway (related to the degradation of proteins for later presentation by the Major Histocompatibility Complex) that correlate with protection against P. falciparum infection after the third vaccination with the licensed vaccine against human malaria RTS-S, suggesting a potential role in mediating protection in vaccinated people that may represent a useful surrogate marker (Table 1). Three recent studies carried out by Van de Berg et al. (2017) (63), Kazmin et al. (2017) (64) and Tran et al. (2019) (65) have provided further insight into vaccine-mediated protection, providing molecular signatures of protective immunity against malaria (Figure 1 and Table 1). By analyzing longitudinal peripheral blood transcriptome and immunogenicity data from
a clinical efficacy trial, in which healthy adults received three
RTS,S doses 4 weeks apart followed by CHMI 2 weeks later, Van
de Berg and collaborators (2017) (63) found that NF-κB and IFN-γ
pathways may induce protection in RTS,S vaccination. On the other hand, a previous study carried out by Kazmin and
collaborators (2017) (64) has suggested that multiple mechanisms
can induce protective immunity against *P. falciparum* given that
specific antibody titers were associated with protection in the
first vaccinated group but not in the second vaccinated group,
where protection was associated with polyfunctional CD4+ T cell
responses. It is interesting to highlight that in this study different
vaccine protocols were used, the first group of 21 volunteers
received three consecutive immunizations of RTS,S/AS01 (RRR
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and at a low cost per base pair. Together with multiplex
platforms (RNA-seq or gene microarrays, among others), high-
resolution techniques (such as AP-SMALDI MSI), and novel
bioinformatic approaches, the way that biological molecules are
sequenced have been revolutionized, facilitating a deeper insight
into parasite-host interactions, transmission, epidemiology, and
as a consequence novel therapeutic targets (Figure 1). Cutting-
edge technologies such as gene editing by CRISPR-Cas9 have also
allowed the discovery and functional characterization of potential
novel vaccine antigens.

The combination of “omics”, computational approaches, and
statistics opens the field of systems biology studies and offers
the possibility to integrate the complex biology of Apicomplexa
and their hosts in a holistic way. This new approach leads to
a better understanding of the mechanisms used by parasites
to avoid host immune defenses and by hosts to balance
parasite actions, leaving behind the reductionist approach, which
analyzes the individual components to infer the behavior of
complex systems.

New technologies such as single-cell RNA-seq platforms
can overcome current limitations of “bulk measures” with
high heterogeneity and analytical variability (69). Whereas the
integration of other “omics” (such as proteomics, metabolomics,
lipidomics, or glycomics) can facilitate and increase the number
of systems biology-based studies, generating new knowledge in
host-parasites interactions and immunology.

DISCUSSION

Next-generation sequencing (NGS) technologies have recently
been applied to many apicomplexan parasites. NGS allow the
reading of simultaneous millions of sequences in a short period
and at a low cost per base pair. Together with multiplex
platforms (RNA-seq or gene microarrays, among others), high-
resolution techniques (such as AP-SMALDI MSI), and novel
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TABLE 1 | Systems biology approaches in the field of vaccinology and immunology applied to *P. falciparum* and *T. gondii*.

| Field       | Species                  | Host or target population | Disease       | Finding                                                                 | Methods                                      | Reference |
|-------------|--------------------------|---------------------------|---------------|------------------------------------------------------------------------|----------------------------------------------|-----------|
| Immunology  | *P. falciparum*<sup>1</sup> | Human                     | Malaria       | Immune signatures: upregulation of genes of the innate response; downregulation of genes involved in phagocytosis and inflammation. Differences in apoptosis genes between symptomatics/ presymptomatics, or uncomplicated malaria | Microarray, computational approaches         | (57, 58) |
|             |                          |                            |               | Immune markers that correlates with severity. Genetic variation associated with severe malaria symptoms and drug-resistance | RNA-seq, computational approaches            | (59)      |
|             |                          |                            |               | Genes differentially expressed and different T-cell activation with parasitemia stages | RNA-seq, gene set enrichment analysis        | (60)      |
|             | *Plasmodium ashfordi*    | Birds, Mice                | Malaria       | Parasite actively regulates host genes related to the immune responses between acute and chronic infection | Transcriptomics, gene set enrichment analysis | (61)      |
|             | *T. gondii*              | Pigs                       | Toxoplasmosis | Parasite actively regulates host genes related to the immune responses between acute and chronic infection | Transcriptomics, gene set enrichment analysis | (61)      |
| Vaccinology | *P. falciparum*<sup>1</sup> | RTS,S vaccinated volunteers | Human malaria | Up-regulation of genes associated Adaptive response. Possible innate genes markers of protection | Transcriptomics, gene enrichment analysis, predictive modeling | (62, 63) |
|             |                          | CSP<sup>2</sup> vaccinated volunteers | Human malaria | Molecular signatures of protective immunity. Differential expression of genes of immune response, protein synthesis, and mitochondrial processes in protected and non-protected individuals | Gene array, predictive modeling, RNA-seq, module correlation network analysis, immunological methods | (64, 65) |

<sup>1</sup>Most recent studies, for further information refer to Tran & Crompton 2019 (66).

<sup>2</sup>CSP: immunization with live sporozoites.
Additionally, systems biology in the context of vaccination could provide novel insights into mechanisms of action of vaccines and molecular signatures involved in protection (Figure 1) to improve design and effectiveness, providing relevant information before vaccine efficacy and safety is assessed in clinical or field trials. These integrative approaches are being incorporated into vaccine development research of protozoa parasites, beyond malarial research, which was pioneering in the field about a decade ago [recently reviewed (66)].

Nevertheless, either vaccine candidates derived from “omics”, computational approaches, or proteins with a validated a specific biological function by genetic manipulation approaches, will still require experimental validation in currently available animal models for the different diseases, evaluating vaccine-specific immune responses, immunoprotection, and safety. In consequence, well-established and unified protocols for animal models to evaluate different diseases caused by Apicomplexa parasites are paramount. Therefore, systems vaccinology combined with experimental validation and subsequent evaluation in animal models can significantly improve the novel design of vaccines against apicomplexan parasites, opening a new era of vaccinology research that could lead to an expansion in licensed products (Figure 1) after decades of significant but slow advances.

AUTHOR CONTRIBUTIONS

The manuscript was drafted by MT and VM-H. Tables and figures were created by MT and AR. All authors contributed to the final version.

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