Delivering on promises? The Impact of Kinetoplastid Genomics on Sleeping Sickness, Chagas Disease and Leishmaniasis

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1. Introduction

Species of the genera *Trypanosoma* and *Leishmania* are protozoan parasites responsible for a series of neglected tropical diseases. The people most affected by these parasites are the poorest living in tropical and subtropical regions of the world.

Two sub-species of *T. brucei*, *T. b. gambiense* and *T. b. rhodesiense*, are the causative agents of human African trypanosomiasis or sleeping sickness. Millions of people living in 36 sub-Saharan countries are at risk of acquiring the disease.[1] Due to increased control over the last decade, the number of reported cases has declined to under 10000 in 2009 for the first time in 50 years.[1] For chemotherapy, only four drugs (suramin, pentamidine, melarsoprol and eflornithine), of which three were developed >60 years ago, and one drug combination therapy (eflornithine/nifurtimox) are available.[1,2] In addition, all drugs have major drawbacks including poor efficacy, significant toxicity, need for parental administration and drug resistance.[3-5]

Trypanosoma cruzi is the aetiological agent of Chagas disease in Latin America. More than 25 million people in 19 endemic countries are at risk of contracting Chagas disease and an estimated 10 million people are infected.[6] Only two drugs (nifurtimox and benznidazole) are available for treatment.[6.7] Both drugs were developed in the 1970s and are only effective in the acute state of the disease (100% effectiveness if given soon after infection).[6] In addition, both medicines have significant side effects, ranging from nausea to life-threatening complications.[7]

Leishmania parasites cause a variety of diseases with different clinical symptoms depending on the species and the immunological status of the human host. Leishmaniasis threatens approximately 350 million people living in 88 countries and currently about 12 million people are infected.[8] The incidence is estimated to be 1-2 million new cases every year.[8] A few drugs (amphotericin B, pentamidine, pentavalent antimonials, miltefosins and paromomycin) are available for chemotherapy.[9] Some of these medicines were developed half a century ago, display considerable toxicity and require parental administration, while the more modern drugs and formulations remain unavailable to most who are infected.

The genomes of *T. brucei rhodesiense*, *T. cruzi* type VI (CL Brener) and *L. major* were sequenced in 2005 and two years later those of *L. infantum* and *L. braziliensis*[10-13], *T. b. gambiense* and most recently *T. cruzi* Type I (Sylvio) have been described in publication.[14,15] There are several more currently being undertaken and the data from some of these has been pre-released into the databases. Whereas the genomes of *T. brucei* and *Leishmania* sp. contain about 8000-9000 predicted protein-coding genes, that from the hybrid genome of *T. cruzi* CL Brenner contained some 1.5 times more, approximately 12000, reflecting large amounts of repetitive DNA such as multicopy genes in tandem array and large multigene families. The Type I *T. cruzi* contained considerably less of this repetitive DNA making the gene repertoire considerably smaller and more in line with the other kinetoplastids. Overall this group of parasites share some 5000 to 6000 conserved core genes, indicating that the proteins encoded by these genes may provide targets for drugs that could be effective against all these parasites.[13,16]

2. Databases for trypanosomatids

The WHO/TDR initiative on *Applied Genomics for Drugs and Diagnostics*, has provided initial support for a series of genome projects which have contributed to an exponential increase in genomic data. Further, *The Pathogenesis and Applied Genomics Committee* of the WHO has promoted the rational use of genomic knowledge towards development of new tools for diagnosis, treatment and prevention of distinct tropical diseases including African and American trypanosomiasis (sleeping sickness and Chagas disease), cutaneous and visceral leishmaniasis.[17] In order to facilitate the analysis of the genomic data, several databases have been developed.

GeneDB (http://www.genedb.org/Homepage) is a curated genome database for pathogenic organisms (parasites and bacteria) and contains genomic information for several trypanosomatids. Currently, this database comprises the datasets of five *Leishmania* species (*L. major*, *L. donovani*, *L. infantum*, *L. braziliensis* and *L. mexicana*) and four *Trypanosoma* species (*T. brucei* (including *T. b. gambiense*), *T. cruzi*, *T. congolense* and *T. vivax*). The trypanosomatid genomic sequence data and annotations are regularly deposited in another

database, TritrypDB (http://tritrypdb.org/tritrypdb/), where they can be incorporate and queried with other datasets. To aid comparative analysis, another bioinformatics resource EuPathDB (http://eupathdb.org/eupathdb/) which includes kinetoplastid, apicomplexan and other eukaryotic pathogens such as giardia, trichomonas, microsporidia and amoeba has also been developed and integrates the genomic data with other platform technologies such as proteomics and transcriptomics where this data is available.[18]

Other databases are collections of protein, enzyme, metabolic pathway, and gene information from different organisms including *T. brucei*, *T. cruzi* and *L. major*. These databases include BRENDA (Braunschweig Enzyme Database; http://www.brenda-enzymes.org/), KEGG (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/), METACYC (Encyclopedia of Metabolic Pathways; http://metacyc.org/), and TTD (Therapeutic Target Database; http://xin.cz3.nus.edu.sg/group/cjttd/TTD_ns.asp) and contain up to several hundred entries for trypanosomatid parasites (see ref. 19 for review on these databases). The medical structural genomics of pathogenic protozoa project (MSGPP; http://www.msgpp.org/) has focussed on producing relevant crystal structures for drug design and has collated the high resolution structural information available for kinetoplastids and other protozoan pathogens for use in drug development while leishbase (http://www.databases.niper.ac.in/LeishBase/) is a structural database devoted to roughly 350 homology based 3D models of *L. major* proteins which have greater than 40% sequence similarity to existing crystal structures. The LeishCyc database (http://leishcyc.bio21.unimelb.edu.au/) is a pathway/genome database for *L. major*.[20] This database was built on the annotated genome sequence of *L. major* and curated on the basis of literature searches, experimental studies and bioinformatic data.[20] The LeishCyc database represents a reconstruction of the *L. major* metabolic network and contains more than 1000 genes that encode for enzymatic or transport reactions.[20] A similar database for *T. brucei*, TrypanoCyc, is currently been developed.[21] These databases will be invaluable resources for detailed comparative studies of trypanosomatids and may lead to the discovery and prioritisation of new drugs for these parasites.

3. Drug target discovery using genome information

The availability of the genome sequences of trypanosomatid parasites has set high hopes for the discovery of new drug targets using a reverse pharmacology approach (Fig 1.). Reverse pharmacology contrasts with traditional drug discovery by starting with the genome as the source of all potential targets and then eliminating through a series of screens those that are unlikely to provide an effective target for drug design. Central to assessment of a protein as a drug target is that it is "druggable" and that its presence is essential for the organism viability. Once this is established, however, there are two fundamentally different methods by which genome targets are identified and developed from the genome. Some targets are identified as homologous proteins to existing drug targets in other organisms. This approach allows for "piggy-backing" of drug development. Piggy-backing is especially effective for targets of anti-cancer drugs, where a conserved enzyme is essential to parasite survival. Once identified, the task is to highlight variation between the structure of the parasite enzyme and the human enzyme that can serve as the basis for drug specificity and modify inhibitors using medicinal chemistry to tailor them for antiparasitic activity without toxicity to the host.

The converse approach involves de novo identification of novel drug targets. This requires the assignment of function roles to the open reading frames (ORF) elucidated by the genome projects. It is generally considered desirable that such targets should specific to the parasite and not the host and should perform an essential and non-redundant function at a pathogenic stage in the life-cycle.

In an attempt to analyse gene functions in trypanosomatids, Subramanian et al. carried out a systematic mRNA ablation by RNA interference (RNAi) of the ORFs of chromosome I in *T. brucei* combined with phenotypic analysis.[22] *T. brucei* was chosen because this parasite is much more experimentally accessible than the related *T. cruzi* and *L. major*. In addition, as African trypanosomes shares about 50-75% of their genes with the other trypanosomatid parasites,[13,16] functional analysis of *T. brucei* genes also provides useful information with respect to *T. cruzi* and *Leishmania* ssp. The RNAi analysis of 210 ORFs of chromosome I revealed that 30% of the ORFs generated a phenotype, mainly involving cell growth, viability and/or cell cycle progression.[22] RNAi against about 12% of ORFs turned out to be lethal.[22]

As many of the ORFs are associated with growth, their gene products may represent potential drugs targets.

In another study, Alves-Ferreira et al. tried to identify potential new therapeutic targets for Chagas disease through *in silico* metabolic pathway analysis.[23] They identified analogous and specific enzymes in *T. cruzi* by comparing the parasite metabolic pathways with the corresponding human metabolic pathways. By focussing on energetic pathways (glycolysis, pentose phosphate shunt, Krebs cycle, oxidative phosphorylation, β-oxidation, amino acid metabolism), lipid pathways and polyamine pathways, they identified several enzymes that are analogous to those of humans.[23] They suggested that many of these enzymes could be potential new drug targets.[23] In a similar study, Capriles and co-workers performed a comparative analysis with the *T. cruzi* genome and the human genome and used comparative modelling techniques to predict 3D protein structures.[24] They were able to identify 397 *T. cruzi* enzyme sequences that are potential candidates for further structure-based drug development.[24]

Trypanosomatid parasites lack *de novo* biosynthesis of purines and depend entirely on the salvage pathway to meet their purine requirements.[25] Because enzymes and transporters of the purine salvage pathway show sufficient differences between trypanosomatids and humans, it is considered as a potential drug target against these parasites.[25] In fact, extensive research on the development of specific inhibitors to target the parasite purine salvage pathway has been carried out over the last two decades.[25] However, based on the genome sequence data, it seems that trypanosomatids possess by-pass mechanisms involving other enzymes and transporters.[26] This poses the question as to whether inhibition of a single enzyme of the purine salvage pathway is enough to kill trypanosomatids or whether inhibition of multiple enzymes by combination therapy would be required.

Using genome sequence data, Camizotti et al. were able to identify and map one locus of *L. major* associated with resistance to two analogues inhibitors of the ergosterol biosynthesis, itraconazole and ketoconazole.[27] They discovered two potential proteins unrelated to the ergosterol biosynthesis that mediate resistance to itraconazole/ketoconazole in wild-type cells after transfection with a cosmid containing the corresponding gene

sequences.[27] This study demonstrated that genome sequences are useful in the identification of genes involved in drug resistance and therefore will be helpful in aiding to develop new strategies for the development of drugs for treatment of trypanosomatid infections.

4. Discovery of vaccine candidates using genome information

The term “reverse vaccinology” was coined for the application of post-genomic methods to providing improved vaccine candidates.[28,29] Reverse vaccinology provides a set of alternative and often faster methodologies when compared with more traditional approaches; where abundant proteins from cultured microbes, often identified as immunogenic using sera from infected or convalescent patients were identified, purified and tested as innocula.

For trypanosomiasis and leishmaniasis the potential for vaccines has always been considered differentially. African trypanosomes are extracellular parasites which replicate in the mammalian bloodstream. On its surface each African trypanosome expresses a coat composed from a single dominant antigen, the variant surface glycoprotein (VSG), and although each trypanosome has a repertoire of thousands of VSGs each representing a different variable antigenic type (VAT), only one variant is expressed at a time. Once a VAT is recognized by the humoral immune system of the host, all parasites expressing it are eliminated, however a small minority switch antigenic type in each generation thereby escaping the immune response and ensuring continued infection of the host (for a post genomic review see ref. 30). This strategy of antigenic variation, consummately utilized by African trypanosomes has been a disincentive for research in this area. In recent years, however, approaches looking focused on conserved and glycosylated epitopes have demonstrated some degree of protection (reviewed in ref. 31).

In contrast to African trypanosomiasis, Chagas disease and the leishmaniases are caused by intracellular parasites. Superficially, the outlook for *Leishmania* vaccines has appeared rather better than for Chagas disease because in at least the case of cutaneous leishmaniasis the practice of “leishmanization”, vaccination with the live organism on an unobtrusive part of the body, has been practiced for many generations and generally confers a longstanding, if non-sterile, immunity to subsequent infection. As a result a great deal of

research focussed on developing a vaccine for leishmaniasis has been undertaken and although the results from challenge models of the disease have not generally proved easy to translate into human protection, several candidates have now been developed including the LeishF recombinant chimeric protein which is now in clinical trials.[32] In contrast, the time course of chronic Chagas disease is often decades from infections before symptoms are manifested. When symptoms do appear, pathology often takes the form of a dramatic monocytic infiltration of the myocardium. However, at this stage of the disease only very low numbers of parasites are normally detectable, whereas autoimmune responses, particularly autoantibodies, are often readily detected. The observations were together construed as a risk that vaccines might induce or exacerbate cardiac disease rather than protecting against it and have limited research in the field until recently, when it clearly demonstrated that pathogenesis is directly attributable to persistence of the parasite. As a result, several groups have begun to consider the potential for a Chagas Disease vaccine (reviewed in ref. 33).

Since the publication of the trypanosomatid genomes a number of studies have applied this knowledge to the identification of vaccine candidates for these parasites using reverse vaccinological approaches as outlined in Fig 2. One starting point for reverse vaccinology is to express every protein encoded by a pathogen's genome and test each for protective qualities without any preconceived bias. This approach was first taken for *Neisseria meningitidis* serogroup B and although laborious showed that the kind of proteins identified in this manner were different to those identified by traditional methods.[34] DNA vaccines provide a mechanism whereby all proteins encoded by a pathogen can be tested without the need for expressing the recombinant protein in bacteria.[35] The whole gene repertoire of a pathogen is cloned into expression plasmid vehicles and inoculated into the model host. The host cells express the parasite protein eliciting immunity and allowing the investigator to evaluate the protection conferred. This approach has been taken for malaria, leishmaniasis, and Chagas Disease yielding some degree of protection in each case and realizing a number of new vaccine candidates.[36-39]

More targeted approaches begin by pre-screening the genomic sequences bioinformatically. Comparative genomics allows highly variable proteins to be eliminated, or

conversely epitopes or antigens which are conserved across a species to be identified. Expression studies working from transcriptomics and proteomics can be used to eliminate proteins that are only expressed in the insect stages. A set of proteins, identified as likely to be antigentic can be discriminated on the basis of surface probably exposure (directed to the plasma membrane surface, GPI-linked, glycosylated or secreted) or on the basis of containing tandemly repeating sequences. Within individual proteins, bioinformatic tools allow for antibody and T-cell epitope prediction and even (HLA-type specific) MHC binding peptides[40] and where these are confirmed experimentally the information is archived at TriTrypDB.

Testing of candidate antigens and epitopes either individually or as multisubunit vaccine is normally undertaken first in mouse models or directly in reservoir hosts. A disadvantage is that protection in model hosts seldom translates into protection in human populations. The advent of humanized mice[41] may provide a more reliable method for evaluating the likely efficacy of human vaccines in future and has been used to investigate a variety of pathogens including hepatitis[42] and Epstein-Barr virus[43] but has not yet been reported to have been used for testing a protozoan pathogen vaccine. Once identified recombinant antigens can be engineered, concatenated and expressed in appropriate vehicles. Oral vaccines are desirable and the technology for using live recombinant viruses such as vaccinia, adenovirus or bacteria such as salmonella as vehicles for expressing protective antigens is well established. Although appealing for vaccination of reservoir hosts and laboratory models because of their ability to be translated quickly from DNA sequence into an appropriate immune response, such vaccines have not been widely adopted and formulations for recent human vaccines in general and anti-parasite vaccines such as the new malaria vaccine and the leishmanial candidate LeishF in particular have taken the traditional form of recombinant antigens introduced with adjuvant.

5. New Diagnostics

The impact from the initial trypanosomatid genome sequences on diagnosis relates mostly to the discovery of new markers for infection, prognosis and cure (Fig 3).

Unfortunately, the genomes projects have also elucidated the remarkable genomic plasticity and intraspecific sequence heterogeneity displayed within these ancient protist lineages. Properties which represent major obstacles for the development of specific and sensitive diagnostic tools and which have highlighted the desirability of re-sequencing from as diverse a range of clinical isolates as possible. Thus, the trypanosomatid genomes have served in formulating and training efficient and effective bioinformatic tools for identifying candidates as molecular markers (Fig. 3). Because of the small numbers of genomes available for whole genome comparisons, however, bioinformatic methods have been limited in their ability to evaluate the likely robustness of these markers and the degree to which they are likely to be undermined by intraspecies heterogeneity in the sequence of each marker.

Focusing exclusively on pathogenic trypanosomatids, major biological differences are observed between species and/or genotypes within a single species in terms of antigenic diversity, infectivity, pathology, and drug resistance, despite their sharing a variety of genes. The dilemma of having distinct biology but considerable conservation at the genomic level led to mapping and gene discovery initiatives based largely in genome analysis.[44] The biology of the human infecting trypanosomatids is very different between species. While *T. brucei* can be readily detected in the blood with a variety of molecular tests, similar detection of *T. cruzi* is quite difficult due to reduced blood parasitemia in the acute phase and can be almost impossible in the indeterminate and chronic phase. Furthermore, in Central and South Americas, *T. cruzi* occurs in sympatry with *T. rangeli*, which also infects humans and wild mammals causing no pathology, but leading to misdiagnosis.[45] Lack of therapeutic options has meant that historically research priority has been driven to searching for new drug and/or vaccine targets as well as to comprehension of the genetic diversity, including antigenic variation, and to the mechanisms related to pathogenesis or drug resistance.[17] The rationale for discovery of new diagnostic markers usually points to a different direction, looking for unique or species-specific alleles that allow for clear and precise detection and recognition of the etiological agent. Such targets should inform the existence of distinct genotypes within a single host, but may not be implicated on the pathogenesis. The physical characteristics of the marker such as nuclear or kinetoplast localization, copy number, genomic arrangement and

intra- and/or interspecific conservancy/variability are the key factors which inform diagnostic utility.

Once the genomes were available, traditional PCR approaches, including nested and multiplex reactions, were tested for a variety of genes. One promising diagnostic methods that has been used for *T. brucei* diagnosis and characterization,[46] as well as for other pathogens, is the loop-mediated isothermal amplification (LAMP).[47] Based on a conserved sequence in the repetitive insertion mobile element (RIME) of the sub-genus *Trypanozoon*, the method is based on a multiplex reaction that enhances the amplification of a stem-loop DNA structure reducing the amplification time and increasing specificity. The isothermal reaction (60-65°C) does not require the use of thermocyclers and the detection of amplification products is visually performed by addition of SYBR Green to the reaction.[46] The system has proven effective for diagnosis even if the detection is based on single copy genes (e.g. PFRA gene[48]), costs less than other DNA amplification-based methods and has a considerable advantage in being easily performed under field conditions.

Since transcription can effectively amplify the amount of target and hence sensitivity particularly in the case of single copy genes, methods detecting RNA molecules such as RT-PCR using total RNA, mRNA or polysomal RNA have been used to assess the presence and viability of the parasites in a quantitative approach. Despite the sensitivity, the method is laborious and time consuming, requiring specialized equipment. Even considering the constitutive transcription observed in trypanosomatids, assessment of RNA molecules from parasites in samples taken from hosts or vectors is hindered by RNA instability, pre- and post-transcriptional RNA modification and distinct life cycle stage transcriptomes. Nevertheless, transcriptomics and proteomics which are inherently postgenomic are key to the development of future diagnostics and already trypanosomatid transcriptomes and proteomes have been described from single life-cycle stage maps to analysis of complex host-parasite interactions.[49] Comparative transcriptomics and proteomics can be synthesized with this information and used to detect species-specific proteins for diagnostic and prognostic purposes, to identify sub-cellular proteomes, virulence determinants and antigens for molecular and serological diagnosis.[49,50]

Linking genome based observation of allelic variation with serodiagnostic methods to produce strain specific diagnostic and prognostic tests has been recently undertaken, where peptides predicted from genome sequence to contain strain specific epitopes were synthesized and used to evaluate sera from infected patients.[51] The gap between molecular and serological diagnostics is also bridged by bead-based flow-cytometric technology which utilizes a variety of probing molecules, including antibodies, antigens (native or recombinant) and oligonucleotides, that are covalently bound to paramagnetic carboxylated microspheres.[52,53] Since each probe can be linked to a bead of distinct color, the technology allows simultaneous (multiplex) detection to up to 500 molecules in a single test and consequently diagnosis, typing, subtyping and even assessment of antigenic diversity or drug resistance markers in a single reaction. An initial study compared this technology favorably with ELISA in terms of sensitivity and specificity when recombinant CRA (cytoplasmic repetitive antigen), FRA (flagellar repetitive antigen) and whole *T. cruzi* cell lysate was used to screen two distinct panels of chagasic patient's sera.[53]

6. Summary and Outlook

The availability of the trypanosomatid genomes has undoubtedly changed the former gene-by-gene approach to search for drug targets, vaccine candidates and diagnostic markers. By unveiling interspecific similarity and intraspecific variability, *in silico* genome analysis has proven to be a revolutionary target-discovery tool. At the present time, discovery and characterization of novel targets for drug design, novel candidates for vaccine development and novel biomarkers for diagnosis are mostly based on gene discovery utilizing bioinformatics based on initial screens which are then followed by laborious, laboratory-based functional analysis and validation. The advent of improved bioinformatic tools, increased numbers of genomes, transcriptomes and proteomes and the deployment of new technologies for high throughput screening, should allow for scaling-up from academic level tests to user-friendly, affordable and precise technologies for even more productive "reverse" drug, vaccine and diagnosis development in the foreseeable future.

7. References

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Fig. 1. Reverse pharmacology pathway – from genomes to lead generation. Figure is adapted from http://www.msgpp.org/flow_diagrams.shtml.

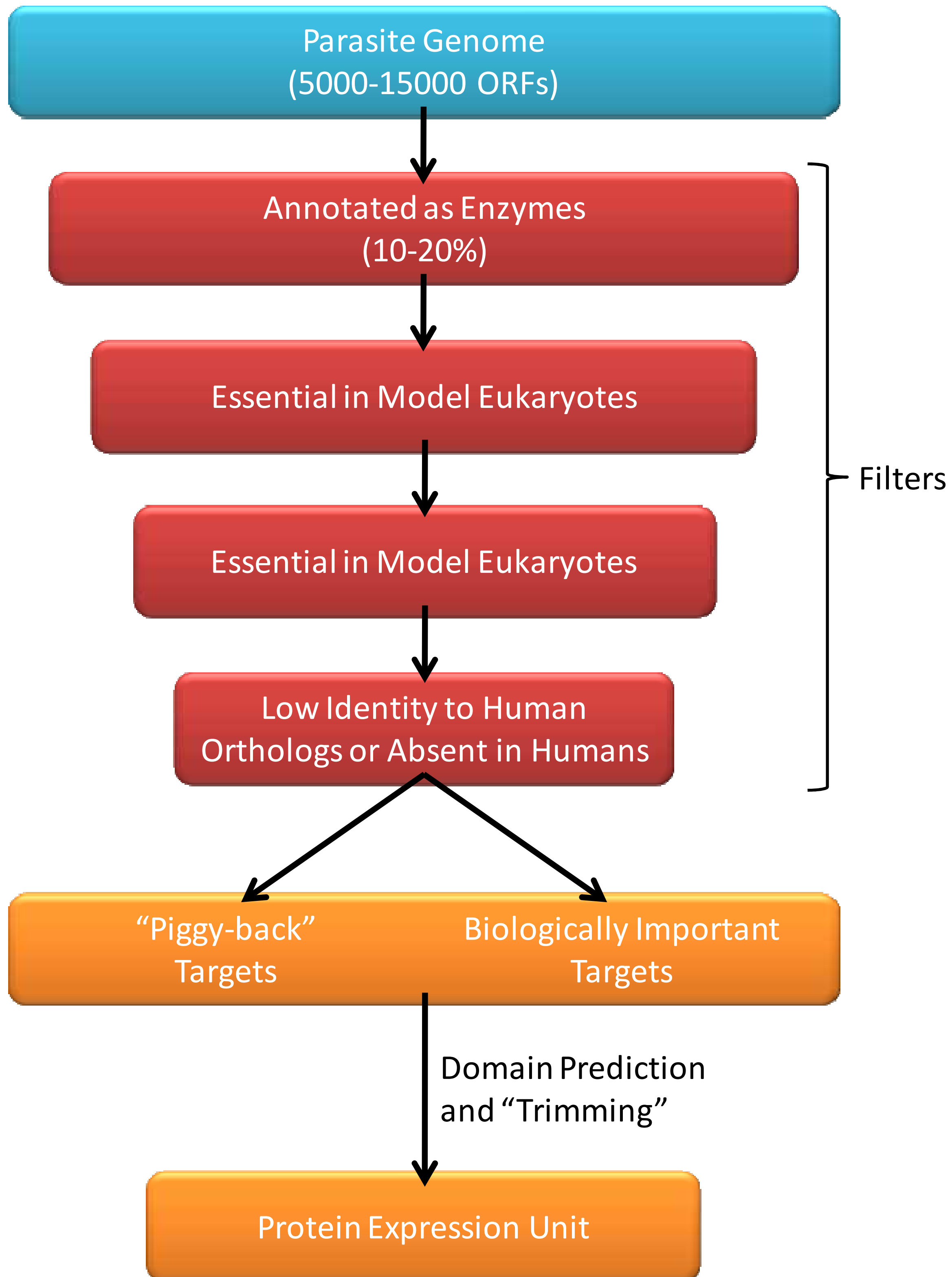

Fig. 2. Reverse vaccinology pathway – from genomes to protection.

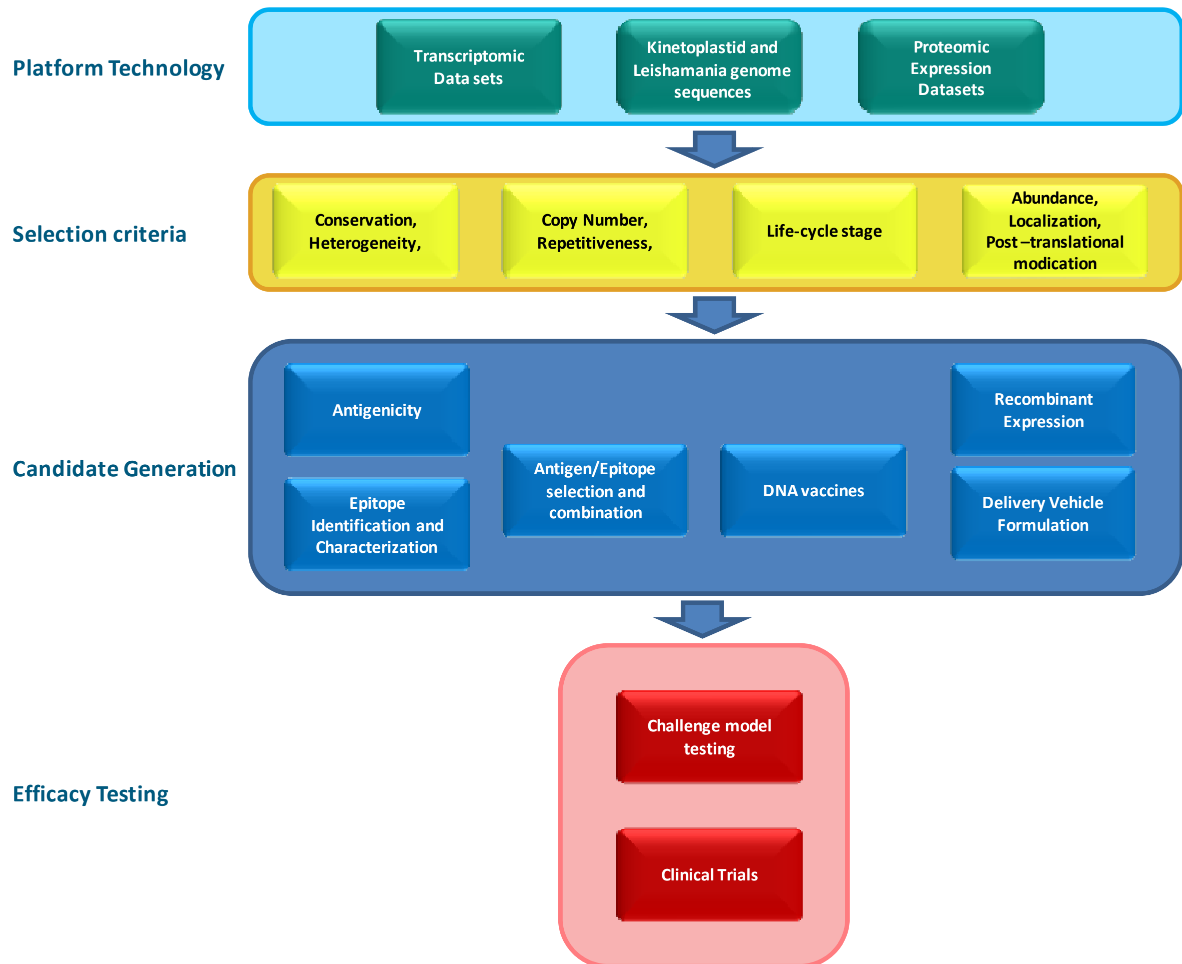

Fig. 3. Reverse diagnostics pathway – from genomes to detection.