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CURRENT OPINION

Genome-Based Bacterial Vaccines: Current State and Future Outlook

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Abstract Genome-based reverse vaccinology (RV) is a multi-step experimental strategy which starts from in silico analysis of whole genome sequences, from which vaccine candidates can be selected by using bioinformatic algorithms to identify putative protective antigens. In this review, we examine the current state of genome-based RV-engineered vaccines and future applications. The first product of genome-based RV is Bexsero®, a vaccine developed for preventing *Neisseria meningitidis* serogroup B infection, and the strategy is currently being used for the development of new vaccines for other obdurate and emerging bacterial diseases. Improved sequencing technologies and the ongoing whole-genome sequence analyses of helminths, protozoa, and ectoparasites also currently serve as a basis for an RV strategy to produce new potential vaccines against eukaryotic pathogens. We also highlight an emerging approach—structure-based vaccinology—that exploits the information derived from the determined three-dimensional structures of vaccine candidates. Regardless, genome-based RV and other vaccine discovery platforms still depend on empirical experimental science to glean, from the hundreds of identified antigens from any one pathogen, those that should be combined to produce an effective vaccine.

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1 Introduction

Vaccination is one of the major health interventions that have had a tremendous impact on reducing mortality and morbidity caused by infectious diseases. The history of vaccination can arguably be dated to Benjamin Jesty (1737–1816) who inoculated his wife and children with cowpox during an epidemic of smallpox in 1774 [1]. Edward Jenner (1749–1823) was likely aware of the use of cowpox and smallpox by country physicians in eighteenth-century England, but his subsequent work represented the first rational and scientific attempt to control an infectious disease. In 1796, Jenner inoculated volunteers with pus scraped from cowpox blisters and showed by subsequent challenges that they were immune to smallpox. Jenner called his procedure *vaccination* (derived from *vaccinia*, cowpox, in turn derived from *vacca*, Latin for cow) [2]. Almost a century later, Louis Pasteur (1822–1895) developed methods for the attenuation of virulent microorganisms as the principal basis of vaccination and produced the first anthrax and rabies vaccines.

All the successful vaccines in use today have been developed on the basis of the principles of a 'conventional vaccinology' and include vaccines against tetanus, diphtheria, poliomyelitis, whooping cough, measles, mumps, rubella, and meningitis [3]. Conventional vaccinology is based on the empirical, trial-and-error identification of protective antigens, often by purification from the pathogen and subsequent analysis of antibody and/or immune cell-mediated recognition. This approach includes using killed or live-attenuated microorganisms, purified, detoxified microbial antigens, recombinant antigens expressed in heterologous organisms or subunit vaccines, which contain identified immunogenic epitopes of protective antigens, e.g., in synthetic peptide and epitope-based DNA vaccine

approaches. However the identification of promising candidates with these methods can be highly time-consuming. Moreover, the conventional approach has limitations in the development of vaccines against pathogens that (1) are antigenically diverse, (2) difficult to cultivate in the laboratory, (3) do not have suitable animal model systems available, and/or (4) are controlled by mucosal or T cell-dependent immune responses [4, 5].

The genome era began in 1995 with completion of the *Haemophilus influenzae* bacterial genome [6], and this paved the way for new developments in vaccinology. Recent advances in rapid sequencing technology and bioinformatics have resulted in an exponentially growing number of published genome sequences. As of March 2013, the complete genome sequences of about 186 archaea, 3,923 bacteria, and 183 eukaryotes have been determined (finished and permanent drafts) and 17,638 are ongoing or incomplete (GOLD Genomes OnLine Database, http://www.genomesonline.org/cgi-bin/GOLD/index.cgi; NCBI, http://www.ncbi.nlm.nih.gov/genome/browse/). Determining whole genome sequences led to the principle of using the genomic information of a microorganism to provide information about the complete antigen repertoire, from which vaccine candidates could be selected by using bioinformatic algorithms to identify putative protective antigens. This novel approach was termed reverse vaccinology (RV) and its use was first published in 2000 by Rino Rappuoli and colleagues for the discovery of potential antigens [7] that has led to the development of a vaccine, Bexsero®/4CMenB, to prevent infection caused by *Neisseria meningitidis* serogroup B (MenB).

The term RV has two different meanings in the context of bacterial/eukaryotic vaccines and viral vaccines. For the former, RV involves the in silico analysis of the microbial genome sequence [8, 9] to identify the surface-exposed proteome (i.e., protein antigens) that a pathogen is potentially able to express and uses this information to predict potential vaccine immunogens. For viral vaccines, the RV strategy involves using the crystal structure of neutralizing monoclonal antibodies (nMAbs) bound to continuous viral epitopes to generate potential vaccines [10]. We find ourselves in agreement with van Regenmortel, who aptly renames the bacterial and viral vaccine approaches as genome- and proteome-based RV (more commonly referred to as genome-based) and structure-based RV, respectively [10, 11].

A common feature of protective viral vaccines is their ability to elicit neutralizing antibodies (nAb) [12]. The RV strategy is metaphorically 'reverse', in the sense that vaccine design starts from known crystallographic structures of broadly protective nAbs bound to pathogen epitopes, instead of trying to generate such antibodies by immunization with defined linear epitopes [10, 11]. Structure-based RV has been applied intensely to the development of HIV-1 [13], influenza virus [14], and human cytomegalovirus [15] vaccines, but overall the approach has not been wholly successful for new viral vaccine development.

Regardless, it should be stressed that both the genome-based and structure-based RV approaches still depend on empirical experimental science to glean, from the hundreds of identified antigens, those that should be combined to produce effective vaccines. In contrast to conventional vaccinology, the main advantage of RV is the speed and reduced cost with which potential candidates can be identified, because there is no requirement for cultivation of the organism [16]. Moreover, RV has the potential to identify all putative protective antigens for a given organism and not just the most abundant ones isolated from culture by conventional methods. It has to be considered that RV was not designed to identify non-protein antigens such as capsular polysaccharides (CPS)—which are components of many successful conjugate vaccines, e.g., *H. influenzae* B, MenC (*N. meningitidis* serogroup C), and Prevenar (*Streptococcus pneumoniae*)—and glycolipids, a promising group of new vaccine candidates [17]. Nevertheless, genome-based RV has become a powerful tool for overcoming the obstacles of conventional vaccinology, leading to the discovery and development of new vaccines for obdurate and emerging bacterial diseases. Improved sequencing technologies and the ongoing whole-genome sequence analyses of helminths, protozoa, and ectoparasites also currently serve as a basis for an RV strategy to develop new potential vaccines against eukaryotic pathogens.

In this review, we summarize the progress of genome- and proteome-based RV, focusing on some projects that have used RV for important bacterial and eukaryotic pathogens. We also highlight some new developments in the application of antigen structural information for bacterial vaccine research.

2 Genome-Based Reverse Vaccinology: The Development of the *Neisseria meningitidis* Bexsero®/4CMenB Vaccine

Neisseria meningitidis is a causative agent of septicemia and meningitis worldwide, leading to high morbidity and mortality particularly amongst young children [18]. The majority of meningococcal infections in European countries are caused by MenB, particularly in countries that have seen the disappearance of MenC disease following the successful use of MenC conjugate vaccines [19]. For MenB, conventional approaches to develop broadly protective universal vaccines have failed, not only because of the poor immunogenicity of the CPS and the structural

similarity of the surface polysaccharides of meningococcal serogroup B to human neural cell adhesion molecule N-CAM [20, 21], but also because of the high degree of variability of major outer membrane (OM) proteins [22]. However, the conventional approach of using OM vesicle (OMV) vaccines, prepared by detergent extraction of meningococcal OM to reduce lipopolysaccharide content and attributable reactogenicity, has been successful in combating clonal MenB epidemics, for example in Cuba [23, 24], Brazil [25], and more recently in New Zealand. In 2004, a 'tailor-made' MenB strain-specific vaccine, MeNZB (strain NZ98/254, P1.7–2,4, ST41/44) [26], was used in a nationwide vaccination program in New Zealand and proved effective in reducing the burden of disease, with a calculated effectiveness of 73 % (95 % CI 52–85 %) [27]. However, the disadvantage of strain-specific PorA-immunodominant OMV vaccines is the lack of cross-protection against other strains capable of causing disease during multiclonal epidemics. Moreover, immune responses to OMV vaccines are very heterogeneous, age-dependent, wane significantly, and often do not rise following booster immunizations. This highlighted the need for new strategies to develop MenB vaccines, including genome-based RV.

Genome-based RV for meningococcal research became feasible with the sequence analysis of the genome of the MenB strain MC58 in 2000 [28] and the experimental process is summarized in Fig. 1 [29]. Whole-genome analysis identified 570 open reading frames (ORFs) that encoded for putative surface-exposed or secreted proteins. All of these were amplified by polymerase chain reaction (PCR) from MC58 and cloned into *Escherichia coli* either as a His-tagged or GST fusion protein. Out of these 570 ORFs, 350 were successfully expressed, and then used to immunize mice. Immune sera were screened by western blot analysis of total cell lysates or OM vesicles to evaluate whether the protein was actually expressed by the bacteria and to determine its localization. Surface expression (or secretion) of the protein was then tested by ELISA and flow cytometry on whole-cell bacteria. Finally, bactericidal assays were performed to evaluate the complement-mediated killing activity of the antibodies, because bactericidal activity correlates with protection in humans [30]. Surface expression was demonstrated for 91 of the proteins and 29 were able to induce a bactericidal response [7]. Natural variation of allelic genes, which is known to lead to the expression of distinct protein variants, must also be considered when selecting proteins for potential inclusion in broadly protective microbial vaccines. Identifying the genetic allelic variants is used to determine the number of different variants of a given protein that are expressed amongst diverse circulating MenB strains. Subsequent analysis of the amino acid sequence variation for each protein can then be used to identify the sequence(s) with the highest potential to ensure broad protective immunity. In addition, stable expression of candidate antigens amongst diverse circulating strains can be used as a selection tool. MenB antigen selection was also refined on the basis of their ability to induce broad protection, either by bactericidal activity and/or passive protection in the infant rat and mouse models [7].

This first universal vaccine developed by Novartis was called 5CVMB (5-component vaccine for MenB or rMenB) and contained *Neisseria* heparin binding antigen (NHBA, previously known as genome-derived *Neisseria* antigen (GNA) 2132) [31], factor H binding protein (fHbp or lipoprotein (LP)2086, previously known as GNA1870) [32, 33], and *Neisseria* adhesin A (NadA, previously known as GNA1994) [34, 35]. In addition, GNA1030 and GNA2091 were selected because they also induced protective immunity, although they did not fulfill all of the assay criteria [36]. GNA1030 and GNA2091 were fused to NHBA and fHbp, respectively, which enhanced immune responses to the individual antigens [36, 37]. When tested against a panel of 85 meningococcal isolates (mainly MenB isolates) that represent the global population of disease-causing strains, murine anti-5CVMB sera were bactericidal against 78 and 90 % of strains when administered with the adjuvants aluminum hydroxide and the oil-in-water emulsion MF59, respectively [36]. 5CVMB has been used in several human trials [38] and when mixed with the New Zealand (NZW) OMV (P1.4 PorA-specific) vaccine, this combination induced bactericidal antibodies against additional MenB strains, thus demonstrating broader vaccine coverage [39]. The NZW OMV was also included to provide additional protection against ST 41/44 clonal complex strains [26, 40] and it appears to provide an immuno-adjuvant effect as shown in animal studies [36] and human trials [41]. The mechanism underlying this adjuvanticity is unclear, but may be related to the immunomodulatory properties of both the vesicle lipid bilayer and inserted OM antigens, such as porins [42]. The contribution and synergism of other antigens capable of stimulating bactericidal antibodies also cannot be excluded.

5CVMB was subsequently renamed 4CMenB (four-component MenB, i.e., the three recombinant/fusion proteins and NZW OMV) and finally Bexsero® [43]. Bexsero® has been used in phase II and III trials involving almost 8,000 infants, children, adolescents, and adults worldwide [39, 44–47] and appears to be well tolerated and generally safe [43]. To estimate further the potential coverage of Bexsero®, a new assay system was developed, which is referred to as the meningococcal antigen typing system (MATS) [48–50]. This assay combines fHbp-, NHBA-, and NadA-specific ELISA data with the genotyping of the PorA

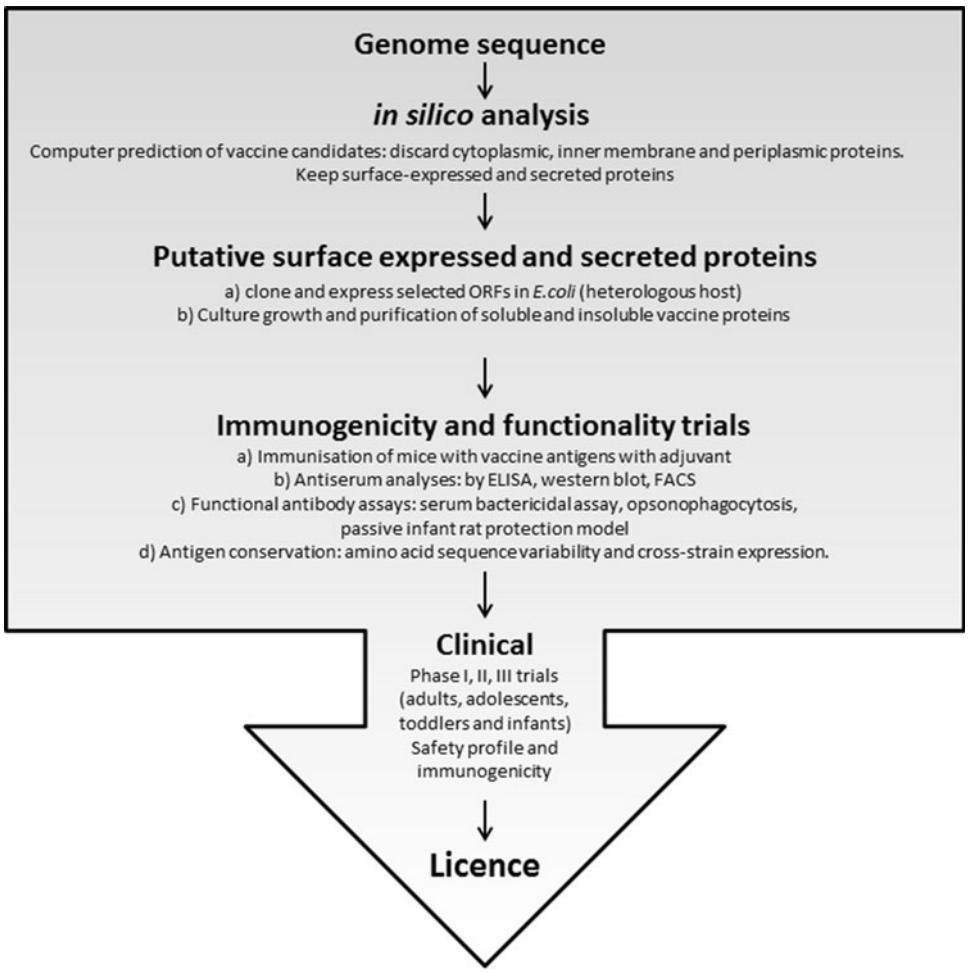

Fig. 1 Genome-based reverse vaccinology: the stepwise experimental approach used to develop Bexsero®/4CMenB from the genome to the vaccine

variant, identified by PCR and sequence analysis. MATS provides a quick and reproducible tool to estimate the level of expression and immunoreactivity of each of the vaccine antigens, in any meningococcal isolate, and it is related to the likelihood that the isolate will be killed by sera from immunized subjects [51]. The relationship between serum bactericidal activity (SBA) and MATS ELISA for each individual antigen has been explored by SBA testing against a panel of 124 strains obtained from meningococcal reference laboratories in Europe, New Zealand, Australia, and the USA, which represented a broad range of amino acid sequence variants in NHBA, NadA, and fHbp [50]. In this study, pooled immune sera from adults were tested for SBA against the 124 strains, and sera from adolescents and from 7- to 13-month-old infants against subsets of 64 and 57 strains, respectively. The authors found that a MATS relative potency (RP) value reflected the ability of strains to be killed in SBA by antibodies to the 4CMenB vaccine and individual vaccine components. Moreover, together with conventional genotyping, it was concluded that the MATS assay was able to describe the capacity of different MenB strains to be covered by the 4CMenB vaccine in infants, adolescents, and adults [50]. More recently, Vogel and colleagues assessed 1,052 MenB strains collected in England and Wales, France, Germany, Italy, and Norway and 108 isolates from the Czech Republic and 300 from Spain (from 2007 to 2008) for the presence of genes encoding for the major antigens in Bexsero® [49]. All of the strains contained at least one gene encoding for one of the antigens and MATS predicted that 78 % of all MenB strains would be killed by post-vaccination sera. The authors identified that 50 % of all the strains and 64 % of the covered strains could be targeted by bactericidal antibodies against more than one vaccine antigen.

MATS data together with the phase II/III trial data served as the basis for submission of Bexsero® for authorization to the European Medicines Agency (EMA). In November 2012, the Committee for Medicinal Products for Human Use (CHMP) of the EMA recommended the granting of a marketing authorization for Bexsero® for use in individuals from 2 months of age and older. The CHMP's 'positive opinion' was sent to the European Commission in support of a marketing authorization (http://www.ema.europa.eu/ema/), which was duly granted

in January 2013 (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:C:2013:053:0003:0009:EN:PDF).

At the same time that Novartis were selecting MenB antigens by genome-based RV, Pfizer (formerly Wyeth) used a conventional approach based on proteome fractionation and protein purification to identify one of the antigens that was also present in 5CVMB. In this approach, multiple sequential fractionations were made of the complex proteome of soluble OM proteins and these fractions were used in animal immunization studies. Candidate fractions were selected on the basis of their ability to induce high levels of bactericidal activity against a number of diverse invasive MenB test strains. This selection identified a single OM lipoprotein, LP2086, which was present in two subfamilies [52]. LP2086 is also known as fHbp [32], and trials of an experimental bivalent vaccine containing two subfamilies of recombinant LP2086 proteins have reached phase II [53].

3 Application of Genome-Based Reverse Vaccinology to Other Bacterial Pathogens

The initial success of genome-based RV in developing a vaccine against MenB served as a proof-of-concept and has encouraged the application of this technology to other bacterial pathogens such as *Streptococcus* spp. (*agalactiae*, *pyogenes*, *pneumoniae*), *Porphyromonas gingivalis*, *Chlamydia pneumoniae*, *Bacillus anthracis*, and others (Table 1). Some of these genome-based RV strategies are outlined below.

3.1 *Streptococcus agalactiae*: Pan-Genome-Based Reverse Vaccinology

Development of a *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) vaccine is an example of multi-genome analysis and screening used as a strategy for identifying potential vaccine candidates against a highly variable pathogen. GBS is a leading cause of life-threatening neonatal infections that can affect at least 1–3 newborns in every 1,000 live births in industrialized countries [54, 55]. Disease is manifested as early-onset disease (EOD), which occurs principally in infants aged 0–7 days and is characterized by respiratory distress, pneumonia, septicemia, and meningitis, and late-onset disease (LOD), which occurs in neonates 7 days to 3 months of age with symptoms of classical features of fever, lethargy, tachypnea, sepsis, and meningitis. Between 36 and 50 % of survivors of EOD/LOD will suffer permanent neurological sequelae, hearing loss, seizures, and mental retardation. Although clinical management has been greatly improved with prenatal screening and appropriate antibiotic regimes, vaccination still represents a practical and attractive strategy for protecting neonates against GBS infection. Vaccines against GBS have been based on tetanus toxoid conjugated CPS, including CPS serotypes Ia, Ib, II, III, and V [56]. Even though these vaccines are likely to protect against the majority of GBS serotypes that cause disease in the USA, they do not offer protection against pathogenic serotypes that are more prevalent in other parts of the world. Thus, a universal protein-based vaccine is highly desirable. The availability of the complete genome sequences of two GBS strains has enabled a genomic approach to identify novel, non-CPS vaccine candidates. Further comparative genomic hybridization revealed a high level of genetic diversity within GBS strains, necessitating the need for multi-genome analysis [57]. To develop a generally protective vaccine, the genome sequences of a further six GBS strains, covering the most prevalent serotypes Ia, II, III, and V, were determined. Multi-genome analysis revealed a core genome of 1,806 genes shared amongst all tested strains and a dispensable genome of 907 genes, which are present or absent in at least one strain [57]. Computational algorithms predicted 589 surface-exposed proteins, of which 396 were core genes and 193 were dispensable genes. Successful expression of 312 candidates was done in *E. coli* and four were found to be protective in a mouse maternal immunization-neonatal pup challenge model [58]. Interestingly, three of the antigens—GBS67, GBS80, and GBS104—were found to be components of covalently linked pilus structures that had not been identified before on invasive GBS [59, 60]. Currently a GBS vaccine is in phase I development (http://clinicaltrials.gov/ct2/show/NCT01193920).

3.2 *Streptococcus pneumoniae*: Comparative Genome-Based Reverse Vaccinology

Streptococcus pneumoniae is the common etiologic agent of community-acquired pneumonia, and causative agent of sepsis, meningitis, and otitis media in young children [61]. On the basis of differences in CPS composition, 94 serotypes are known. Serotypes vary in the extent to which they are carried in the nasopharynx and the degree to which they are recovered from different disease states [62, 63]. A multivalent unconjugated CPS vaccine (Pneumovax 23) and a conjugated CPS-based vaccine (7-valent or 13-valent Prevenar) are available, which are highly protective against serotypes included in the formulation [64–67]. However, the variable epidemiological distribution of serotypes among developed and non-developing countries [68], the poor immunogenicity of unconjugated CPS-based vaccine, and increasing resistance to antibiotics such as penicillins, cephalosporins, and macrolides are some of the reasons for developing a serotype-independent vaccine based on protein antigens [69]. Furthermore, following the introduction

Table 1 Examples of the genome-based reverse vaccinology approach in infectious diseases research

Pathogen	Vaccine developed or antigens identified	References
Bacteria		
Neisseria meningitidis	4CMenB (Bexsero®) vaccine	See text
Actinobacillus pleuropneumoniae	58 conserved genes coding for OMPs or lipoproteins as potential vaccine candidates	[101]
Anaplasma marginale	21 OMPs capable of inducing bovine IgG2 opsonizing antibodies	[102]
Brachyspira hyodysenteriae	Conserved proteins P-H7, P-H17, P-H34, P-H42	[103]
Brucella spp.	Using VaxiJen (web-based vaccine design program based on reverse vaccinology), identified O-sialoglycoprotein endopeptidase; 32 OMPs (including Omp2b, Omp25, Omp31-1, TonB-dependent receptor proteins), adhesins or adhesin-like proteins (including flagellar hook proteins FlgE and FlgK)	[104]
Chlamydia pneumoniae	LcrE antigen	[76]
Edwardsiella tarda	Flagellar protein FlgD	[105]
Ehrlichia ruminantium	11 proteins eliciting an in vitro cellular immune response	[106
	LMW proteins <20 kDa	[107]
Escherichia coli	FdeC (factor adherence *E. coli*)	[108]
	Protective antigens (ECOK1_3385, ECOK1_3457, ECOK1_3374, ECOK1_0290, ECOK1_3473 c1275, c5321, c0975, ecp_3827)	[109]
Haemophilus parasuis	Three ABC-type transporters (OppA, YfeA, and PlpA) and 1 curli protein assembly (CsgG)	[110]
Leptospira serovars	Cation efflux system membrane protein (czcA) and four subunit peptides	[111]
Leptospira interrogans	226 genes identified as vaccine candidates	[112]
Pasteurella multocida	PlpE surface-exposed protein	[113]
Porphyromonas gingivalis	PG32, PG33 (OmpA-like proteins)	[114]
Streptococcus agalactiae	GBS322 (SAG0032, Sip protein), GBS67 (SAG1408), GBS80	[58]
	(SAG0645), GBS104 (SAG0649) proteins	[59]
	HMW pilus-based vaccine	[94]
Streptococcus pneumoniae	Protective antigens Sp36, Sp46, Sp91, Sp101, Sp128/130 (cell wall anchor)	[71]
	RrgB321 (fusion protein of three RrgB variants)	[73]
Streptococcus pyogenes (GAS)	Recombinant pilus proteins Cpa, MI_128, and MI_130	[115]
	Protective antigen Spy0416	[116]
Streptococcus sanguinis	28 lipoproteins and 19 cell wall-anchored proteins	[117]
Streptococcus suis	RTX family exoprotein A (RfeA), epidermal surface antigen (ESA), immunoglobulin G (IgG)-binding protein (IBP), suilysin (SLY)	[118]
Ectoparasite		
Rhipicephalus microplus (cattle tick)	176 membrane-associated and 86 secreted soluble proteins, peptide 1 from antigens 2, 3, and 4 react with polyclonal antisera	[91]
Protozoa		
Cryptosporidium parvum, C. hominis	Cp15, profilin, apyrase	[77]
Leishmania major and *Leishmania infantum*	19 proteins identified as common antigens to both organisms	[80]
Leishmania spp.	Not mentioned	[79]
Plasmodium spp.	MalVac database of vaccine candidates	[82]
Theileria parva	Tp2, containing multiple CTL epitopes	[119]
Helminths		
Echinococcus granulosus	Tegumental membrane protein enolase	[78]
Schistosoma japonicum	Th1 cell epitopes from secreted and transmembrane proteins	[81]

OMP outer membrane protein, *LMW* low molecular weight, *HMW* high molecular weight

of the conjugated CPS-based vaccines, the pneumococcal population structure has shifted as a result of "capsule serotype replacement", leading to a greater prevalence of non-vaccine serotypes in carriage and disease after vaccination [70].

Wizemann and colleagues [71] first applied a microbial genomic approach to identify novel vaccine candidates against *S. pneumoniae*. From the genome sequence data of a serotype 4 pneumococcus, the authors identified 130 ORFs encoding putative surface proteins or with similarity to predicted virulence factors. Form these data, 108 recombinant protein antigens were produced and six candidates were discovered owing to their ability to elicit a protective immune response in mice. The utility of comparative genome hybridization of 30 pneumococcal isolates led to the discovery of pili in *S. pneumoniae* and pilus subunits are now being tested as vaccine candidates in mouse models [72]. However, to date, a fusion protein containing the three RrgB variants [73], the major backbone subunit of pilus 1, only has the potential to cover approximately 30 % of all pneumococcal strains and is unlikely to be included in its present form in any multivalent protein antigen-based pneumococcal vaccine.

3.3 *Chlamydia pneumoniae*: A Genome–Proteome-Based Reverse Vaccinology Approach

Chlamydia pneumoniae is an obligate intracellular pathogen and a common cause of respiratory tract infections worldwide. In addition to causing acute infections, it has been associated with chronic lung processes such as asthma and chronic obstructive pulmonary disease (COPD) [74]. Despite the prominence of *C. pneumoniae* in a wide variety of serious human diseases there is currently no effective vaccine available. Chlamydial infections are treated by antibiotic therapy; however, a protective vaccine preventing severe sequelae would constitute an acceptable short-term goal to control infections caused by *Chlamydia*. The biological complexity of *Chlamydia* and the existence of multiple serovariants provide considerable challenges to developing a vaccine based on modern approaches such as genome-based RV. In an attempt to analyze the surface protein organization in *C. pneumoniae*, a combined genomic-proteomic approach has been used [75]. This identified 53 surface antigens, five of which were tested as vaccine candidates in a mouse model. One candidate, LcrE, was selected by this process as a protective antigen [76]. LcrE is the putative lid of a type III secretion system in *Chlamydia* and vaccination with LcrE resulted in an increase in both the numbers and levels of activation of CD4+ and CD8+ T cells with expression of IFN-γ and TNF-α. Expression of these two cytokines correlated with protection from intranasal challenge with a homologous *C. pneumoniae* strain [76].

4 Application of Genome-Based Reverse Vaccinology to Eukaryotic Pathogens

Following the successful application of RV to the discovery of vaccine candidates against prokaryotic pathogens, the same strategy is being used for eukaryotic pathogens (Table 1). However, such vaccine studies are especially challenging, given the multifaceted life cycles of these multicellular organisms. At present, RV has been employed for a number of important helminths, protozoa, and ectoparasites that cause human disease. These include *Cryptosporidium parvum*/*Cryptosporidium hominis* [77], *Echinococcus granulosus* [78], *Leishmania major* and *infantum* [79, 80], *Schistosoma japonicum* [81], and *Plasmodium* spp. [82].

Cryptosporidium is a protozoan parasite that belongs to the family Apicomplexa. It causes acute gastroenteritis and diarrhea, both in immunocompromised and immunocompetent people [83]. *Cryptosporidium* is an emerging pathogen that excessively affects children in developing countries, but disease incidence is also increasing in industrialized countries, largely as a result of outbreaks in recreational water facilities [84]. Genome-based RV using the published genome sequences of *C. parvum and C. hominis* [85, 86] identified three antigens—Cp15, profilin, and a *Cryptosporidium* apyrase—that could induce specific and potent humoral and cellular immune responses [77]. Profilin is a potent agonist of the innate immune system through its recognition by Toll-like receptor 11, and a homologue in the parasitic protozoan *Toxoplasma gondii* contributes to invasion. In addition, both the *Cryptosporidium* apyrase and Cp15 could also be associated with the invasion process.

Echinococcus granulosus (dog tapeworm) is a cyclophyllid cestode and the causative agent of cystic hydatid disease, an endemic helminthic disease, which affects sheep, cattle, dogs, and humans. It has a two-stage life cycle existing as worms in the gut of infected dogs, which are the definitive host, and as cysts in herbivores and humans, which represent intermediate hosts. The disease is wasting and can be life-threatening due to the development of hydatid cysts within the internal organs of the infested person [87]. Whole-genome sequencing of cestodes started in 2004 and includes the aetiological agents of alveolar echinococcosis (*Echinococcus multilocularis*), cystic echinococcosis and neurocysticercosis (*Taenia solium*) as well as the rodent-hosted laboratory model *Hymenolepis microstoma* [88] and has now been completed [89]. So far, only a few potential vaccine candidates have been discovered. The oncosphere antigen Eg95 was successfully developed into an applicable vaccine for *E. granulosus*, but with the limitation that Eg95 is species-specific. Applying genome-based RV to *Taenia asiatica* (after *T. solium and Taenia saginata*, the third major causative organism of human taeniasis), Gan and colleagues [78] identified enolase as a membrane-bound vaccine candidate in the worm. Enolase from *T. asiatica* is localized on the tegumentum of the worm and a homologue expressed sequence tag (EST)

from the EST database was also discovered in *E. granulosus*. EgEnolase was cloned and expressed in *E. coli* and the purified recombinant protein was recognized specifically by sera from a patient infested with *E. granulosus*. EgEnolase could be visualized in the tegument of the protoscolex and bioinformatic analysis revealed 14 linear B cell epitopes and 6 cytotoxic T lymphocyte (CTL) T cell epitopes, suggesting its strong immunogenicity and potential as a vaccine candidate.

Development of the first large-scale genomic analysis of the medically significant tick *Ixodes scapularis* (black-legged tick, commonly known as a "deer tick", which can transmit the organisms responsible for Lyme disease, anaplasmosis, babesiosis) and the nearly completed genome of *Rhipicephalus microplus* (an obligate hematophagous parasite of domestic and wild animals that serves as vector of infectious agents lethal to cattle) has allowed genome-based RV to be considered for the design of new anti-tick vaccines. Using a combination of functional genomics and in silico prediction with VaxiJen (an alignment-free approach) [90] a total of 176 putative membrane-associated and 86 putative secreted soluble proteins were identified in *R. microplus* [91]. These proteins were selected on the basis of their expression in all life stages and adult tissues. Protein selection was then refined on the basis of putative membrane association. Thus, five proteins (named Antigen 1 to 5) were chosen and three of them were identified that reacted specifically with antisera from mice immunized with crude *R. microplus* midgut extracts. Currently, further promising candidates are being expressed for vaccine trials (Table 1).

5 Conclusion and Perspectives

Genomics has fundamentally changed the way we approach vaccine development. A combination of the ever-expanding numbers of prokaryotic and eukaryotic genomes, the sophisticated computer programs that can predict gene and protein function, cellular localization and homology, and advances in methods for biochemical and structural analyses of proteins has been used in intensive research programs to develop 'next-generation vaccines' against organisms once thought intractable. The first success story for RV is Bexsero®; yet, despite more than a decade of resource-intensive development using state-of-the-art technologies, which has generated an outstanding body of published work, even this vaccine may not represent the 'universal' vaccine against MenB. A possible flaw in the RV strategy for MenB is that of the 570 ORFs that encoded for surface-exposed or secreted proteins, 350 were successfully expressed. Given that Bexsero® is likely to cover 73–87 % of circulating MenB strains in five European countries [92], could it be that the other 220 ORFs contain antigens that might be important for increasing vaccine coverage? Monitoring of antigen expression will become critical following the introduction of Bexsero® and indeed other multi-component vaccines.

It is not surprising that serologically immunodominant antigens with vaccine potential are under immune pressure and highly variable. The development of many bacterial vaccines has been hampered by amino acid sequence variability within such protective antigens. Clearly a need still exists for interrogation of other, perhaps more conserved antigens, with vaccine potential for inclusion in the next-generation MenB vaccines. The degree of variability may be the key here: complete conservation of amino acid sequence suggests that such antigens are probably not well exposed to the immune system, and for this reason are unable to elicit protective immunity. Minor variation suggests some degree of immune pressure, and although such antigens are unlikely to be immunodominant, they can possibly act synergistically and contribute to protective immunity.

Possibly, a structure-based vaccinology approach could be used to revisit immunodominant antigens once thought too variable for broad protection. In order to overcome this obstacle for the development of a second-generation of MenB vaccines, a structure-based vaccinology approach has been suggested, in which multiple immunodominant epitopes that induce immunity against different antigenic variants have been 'grafted' onto a single molecule. This approach has been tried with fHbp [93], which is known to have more than 300 variations in amino acid sequence, and these differences can be organized into three separate groups of antigenic variants that do not induce cross-protective immunity. In order to generate a single antigen capable of inducing immunity against potentially all sequence variants, Scarselli and colleagues [93] rationally designed, expressed, and purified 54 different mutants of fHbp and tested them in mice for their ability to induce protective antibodies. From this panel of 54 mutants, the authors identified a lead candidate fHbp mutant G1, which induced cross-reactive bactericidal antibodies. Then, by combining the knowledge of the epitopes recognized by variant-specific MAbs and the three-dimensional crystal structures of the wild-type fHbp variant 1 and mutant G1, the authors successfully engineered a single recombinant chimeric molecule that displayed two immunodominant domains capable of inducing cross-protective antibody responses against a panel of fHbp antigenic variants of MenB [93].

A structure-based vaccinology approach has also been used to design a fully synthetic protein with multivalent protection activity, based on the hypervariable GBS cell-surface type 2a (BP-2a) pili [94]. In GBS pili, the backbone

subunit is present in six structurally similar, but immunologically different variants. In this study, the three-dimensional structure of one of these BP-2a variants was determined and the authors demonstrated that protective antibodies specifically recognized one of the four domains that constitute the protein. On the basis of this information, a synthetic protein was constructed that contained the protective domain from each of the six pili variants and this chimera was able to provide broad protection in mice infected with all of the type 2a pilus-carrying strains [94]. This novel strategy potentially provides a transferable technology platform for the development of vaccines against other bacterial pathogens.

Other questions regarding the use of Bexsero® will only become apparent when the vaccine is introduced into the routine immunization schedule: fundamentally, will it reduce cases of MenB disease? Also, as protein antigens included in Bexsero® are not strictly specific for serogroup B, will this recombinant vaccine have the potential to cross-protect against non-B serogroup meningococci? What are the longer-term issues relating to immunological memory and persistence of bactericidal antibody responses? Will it impact on carriage and transmission and generate herd immunity? Or, will it lead to the selection of strains not covered by the vaccine, as we now see with pneumococcal conjugate vaccines? Should a second-generation vaccine be developed without the OMV component, which introduces a higher antigenic complexity, given the composition of the OMV proteome [95]? OMVs are likely to contribute to reactogenicity and its removal would reduce the incidence of fever following vaccination. Recently, two randomized trials have shown that vaccination with 4CMenB concomitantly with routine infant and childhood vaccines increased reactogenicity, generally manifested as fever (≥38.5 °C) and was possibly associated with febrile seizures in two vaccines [47]. One could also envisage that the identification of additional OM antigens capable of inducing broad cross-protective antibodies, whether by genome-based RV vaccinology, immuno-proteomics [96, 97], or other selection platforms [98, 99], could be used to develop a second-generation vaccine based within a synthetic membrane that mimics the OM, without the antigenic complexity introduced by redundant proteins not associated with protection. But what this makes clear is how little we really know about the structural biology of the OM.

Another question for the introduction of vaccines developed from genome-based RV is likely to be cost-effectiveness: in a recent analysis for Bexsero® introduction in England [100] with an estimated cost of GB£40 (US$65), given the current rates of infection, the quality-of-life gained was estimated to cost GB£100,000 (US$160,000), which points to a vaccine unlikely to be cost-effective. Introduction of this (and other RV-generated vaccines) will depend on private negotiation and decision-making that must take into account cost. All of these questions are likely to be levelled at many of the other vaccines currently 'in the pipeline'.

Finally, despite the sophistication of new vaccine discovery platforms including genome-based RV, vaccine development still remains to a large extent one of empirical selection, of a trial-and-error testing of candidate components for their ability to induce protective immune responses. Arguably and fundamental to these vaccine design strategies is a complete understanding of pathogen biology, from the genome to the complex networks of theoretical and practical *-omics*.

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