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Revivals in Reverse Vaccinology: A Genomic Perspective.

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ABSTRACT

Reverse Vaccinology (RV) combines bioinformatics and biotechnological techniques to develop a vaccine. It involves initial screening of the whole genome for the prediction of possible antigens which includes extensive in vitro analysis leading to the development of a new vaccine. This technique has reduced considerable amount of work, time and cost in involved in the development of a vaccine to a greater extent. The review analysis the application of RV in major bacterial pathogenic diseases. The availability of whole genome sequences of many human pathogens, RV could be implemented to develop a much better vaccine covering almost all the possible strains and serotypes of each pathogen. However, RV is lacking in the development of vaccines against viral infections, though their whole genome sequences are available.

Key words: Reverse Vaccinolgy, vaccine, antigen, genome sequence, bioinformatics

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INTRODUCTION

Vaccine is a biological preparation that improves immunity to a particular disease. A vaccine typically contains an agent that resembles a disease-causing microorganism, and is often made from attenuated or killed forms of the microbe, its toxins or one of its surface proteins. Vaccination has been the most effective interventions to decrease mortality and morbidity due to infectious diseases in the history of mankind. The earliest attempts to develop a vaccine were for smallpox. The first small pox vaccine was developed by Edward Jenner in 1796 after anecdotal observation of milkmaids infected by cowpox were protected against smallpox. Jenner demonstrated protection against smallpox could be achieved by deliberately inoculating people with small doses of cowpox pustules [1].

Reverse Vaccinology (RV) represents a revolution in immunology and a milestone in biotechnology. With the advancements in the genome sequencing techniques, there has been an exponential increase in completely sequenced bacterial genome from very few to 2615 till date (http://www.ebi.ac.uk/genomes/bacteria.html). This has led to the identification of number of novel antigens which were previously unrecognized by conventional vaccinology due to deprived or null expression in vitro and impossibility of culturing the pathogen. Unlike the conventional vaccionology, RV enables to identify surface-exposed proteins starting from the genome rather than the microorganism [2-3].

The aim of this work is to critically assess some of the representative RV technique currently implemented for the development of vaccines in various pathogens. The review covered literatures on key issues such as documentation and application of various bioinformatics approaches which has aided the development of vaccine by utilizing RV for major pathogens. The review highlights the importance of RV in vaccine development and demands for extending the technique for other pathogens as well.

RV technology work process

Starting with the screening process either through RV or pan RV or comparative RV, the potential ORF's are identified using databases and computer programs included in Wisconsin package version 10.0 [Genetics Computer Group (GCG); http://www.accelrys.com]. Homology searches against a database using BLASTX, BLASTN and TBLASTX programs were done to discard ORF's coding for known antigens and selecting the remaining ORF's for further analysis. BLAST, FASTA, MOTIFS, FINDPATTERNS and PSORT in addition to the ProDom, Pfam and Blocks databases are used to screen the putative proteins and to predict features typical of surface-associated proteins. The protein features includes transmembrane domains, leader peptides, homologies to known surface proteins, lipoprotein signatures, outer membrane anchoring motives and host-cell binding domains. The next step involves cloning of the selected genes into suitable vectors. Once these genes are expressed, they are purified and are used to immunize the mice. The antibodies produced against the immune sera are analysed for their effectiveness against the antigen. Finally, the selected antigens enter the multiple stages of clinical trials. **Table 1** discloses the usage of RV technique for the development of vaccines in most prominent pathogens.

FIRST RV VACCINE

RV was first implemented on serogroup B of *Neisseria meningitides* which causes meningitis and sepsis in children and young adults. The bacterium is classified into 13 serogroups on the basis of chemical composition of the capsular polysaccharides. Out of these serogroups A, B, C, Y and W-135 cause disease in humans. The polysaccharide based vaccine development approach was successful for serotype A, Y and W-135 but this method failed in the case of MenB. Since, MenB capsular polysaccharide is identical to a widely distributed human carbohydrate (α[2→8]N-acetyl neuraminic acid or polysialic acid), which being a self-antigen might elicit autoantibodies.

The availability of whole genome sequence of MenB, led to the identification of putative ORF's. Those which had coded for surface exposed proteins were only selected. The further screening process was carried out based on their expression in E.coli and bactericidal activity. This had let to the identification of five antigens (GNA3132 (nmb2132), GNA1030 (nmb1030), GNA2091 (nmb2091), GNA1870 (nmb1870), NadA (nmb1994)) which were to be included in the vaccine. These 5 components were expressed as 3 recombinant proteins with 2 fusion proteins as shown in **Fig.1**. NadA was found to have variable functions such as cell

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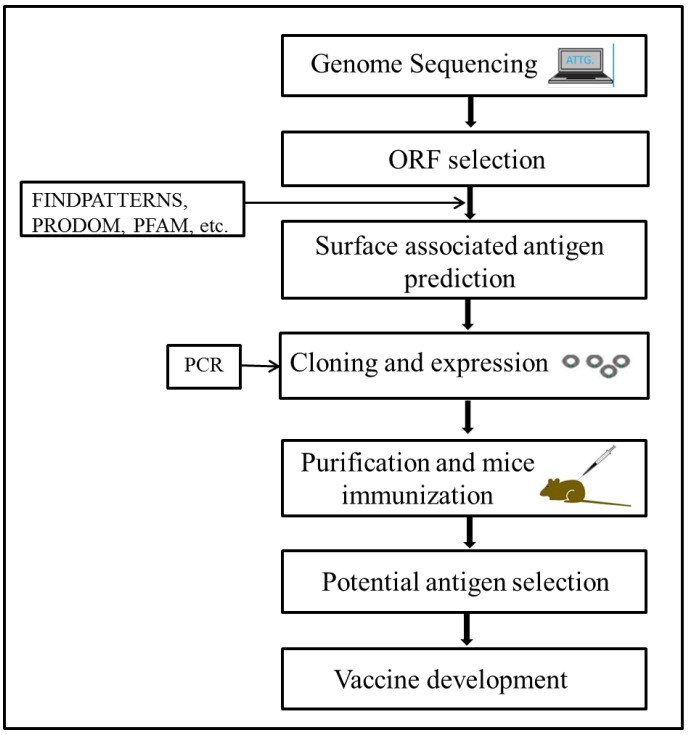
adhesion, invasion, induction of pro-inflammatory cytokines and involved in interaction with b-1 integrins. GNA1870, named as fHbp (factor H binding protein) and nhbA (Neisserial heparin binding antigen) as it functions as a receptor for the human complement regulatory protein factor H and it is also found to be associated with heparin binding.

Figure 1: General process of RV



Initially from the whole genome sequences available, ORF's are traced out related to the known antigens. Using various packages, surface-associated antigens are selected. These antigens are tested for various properties such as transmembrane domains, leader peptides, homologies to known surface proteins, lipoprotein signatures, outer membrane anchoring motives and host-cell binding domains before choosing the possible putative antigen. Once the antigen is chosen, it is cloned and expressed in the bacterial culture followed by mice immunization as a part of pre-clinical trials. Finally western blot, ELISA and FACS techniques are used to select the putative antigen before the vaccine is developed and entering the clinical trials.

RV FOR ANTHRAX

Anthrax is caused by *Bacillus anthracis* primarily in sheep and cattle which can be transmitted to humans through various sources and hence has been widely used as a bioweapon. Having caused widespread attacks, there has been a call to develop a potential vaccine for this disease.

Using the concept of RV *In silico* studies have been proposed to predict the putative vaccine candidates. Initially the genome of *B. anthracis* was extracted from JCVI CMR (TIGR). This sequence was screened using SDSC biological workbench and the sequence with the least identity of 24.73% with accession number ZP_05214185.1 was employed. MAPPP (MHC-I Antigenic Peptide Processing Prediction) was utilized to predict the binding and proteasome cleavage peptides for those sequences having less E value and less identity which were in turn determined using EMBOSS antigen. From the whole sequences, 14 antigenic determinants were identified. The LCV values for all the antigens chosen were calculated and the one with greater LCV value was considered. The findings were confirmed with MAPPP results. The epitope predicted "TSLVVEVVVESK" was modelled and energy minimized using Discovery studio 2.5. The energy of MHC I molecule was -164.664 k cal/mol. Docking of MHC I using CDocker with the chosen epitope produced a binding energy was 169.117 k cal/mol. Having the least energy, this epitope was successfully chosen as the best vaccine candidate for further analysis (**Fig.2**). [5]

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Table 1: Progress in RV for major diseases

Disease/causative organism	RV	Progress	Why RV?	Reference
Meningitis	Yes	Vaccine launched in market	MenB capsular polysaccharide is identical to a widely distributed human carbohydrate (α[2→8]N-acetyl neuraminic acid or polysialic acid), which being a self-antigen might elicit autoantibodies	[4;12;14;15]
Anthrax	Yes	Vaccine candidates have been identified	Conventional vaccinology techniques have not been successful in eradicating all the strains of anthrax bacteria	5
Tuberculosis	Yes	Vaccine candidates have been identified	No potential vaccine exists for tuberculosis	6
Malaria	Yes	A detailed database has been developed on the vaccine candidates predicted using RV	Conventional vaccines failed due to lack of information on immune responses to the parasite	[7]
Herpes	Yes	UL26.5 was identified as the potential vaccine target; experimental studies are to be carried to test its efficacy	Antibodies against glycoproteins gB and gD showed induced protection in animal models but failed in humans	[10-11]
Leishmaniasis	Yes	Vaccine candidates have been identified for various species of the disease causing organisms	Factors like virulence, genetic differences between the species and coexistence among various forms of the disease	[8-9]
Porphyromonas gingivalis	Yes	From the whole genome sequence, 120 genes were selected and expressed in *E.coli,* followed by screening with *Porphyromonas gingivalis* antisera. The Fimbrial antigens were well known for their immunogenic boosting activity. Combination of these with CPS glycoconjugates helped to broaden the spectrum, were found to be ambitious vaccine candidates	The available surgical and nonsurgical treatments could not be made available due to insurance issues and use of such antibiotics without proper diagnosis was undesirable. Hence, there was a need to develop a proper and safe vaccine.	[16-17]
Cancer	No	Only hypothesis	-	-
HIV	No	Only hypothesis	The subunit vaccines developed through viral envelope were successful and tested in phase I and phase II trials, in phase III it was observed that the vaccine did not neutralise the divergent viruses extracted from patient	[18]
Echinococcus granulosus	Yes	The tegumental membrane protein enolase has been deduced as a potential vaccine candidate	The oncosphere antigen Eg95 is species-specific and thus, the vaccine developed from this antigen failed to be effective on 7 different genotypes of *E. granolosus*	[19]
Staphylococcus aureus	Yes	58 genes were chosen based on i) surfaced bound proteins that possess LPxTG anchoring motifs, ii) proteins known to interact with host extracellular ligands and iii) secreted proteins playing a role in immune evasion/modulation. Out of these, 25 genes encoded surface bound proteins and 13 of them were secreted proteins	The MRSA reduces the choice of effective antibiotics for prevention and treatment	[20]

Streptococcus pneumoniae	Yes	From the initial 140 antigens, 18 were selected for animal studies based on various in vitro assays. Out of which 4 showed protection against lethal sepsis. Any lead vaccine was intended to contain PcsB and StkP for protection against all serotypes of pneumococcus. Lipoate protein ligase (Lpl) and ClpP protease, showed reduced mortality in animal sepsis model	Full coverage vaccines, made of capsular polysaccharides as potential antigens, were not completely available due to more than 90 different serotypes	[21,22]
Chlamydia pneumonia	Yes	FACS binding assays 53 proteins of *C.pneumoniae* strain CWL029 were shortlisted that had the ability to bind to chlamydial cell and 28 of 53 antigens were identified on 2DE maps. 6 induce antibodies in a dose-dependent manner in mouse in-vitro cell cultures. These same antigens were experimented on hamster model. 2 genes *cutE* and cpn0420 were able to follow 3 critera: i) prevention of *C.pneumoniae* induced death, ii) reduction of lung disease, and iii) elimination of *C.pneumoniae,* while all other genes failed in one or more criteria	None of the antigens predicted could allow protection like that of natural immunity after asymptomatic low-level *C.pneumoniae* infection	[23,24,25]
Streptococcus pyogenes	Yes	The SpnA surface-associated protein was deduced as putative candidate showing important role in virulence produced by *S.pyogenes*in	-	[26]

Table 2: Sequence identity of the shortlisted genes

Sequence Identity	Accession number	Gene ID
22.5%	NP_216495.1	15609116
24.53%	NP_215297.1	15607923
23.171%	3CXY	206581993

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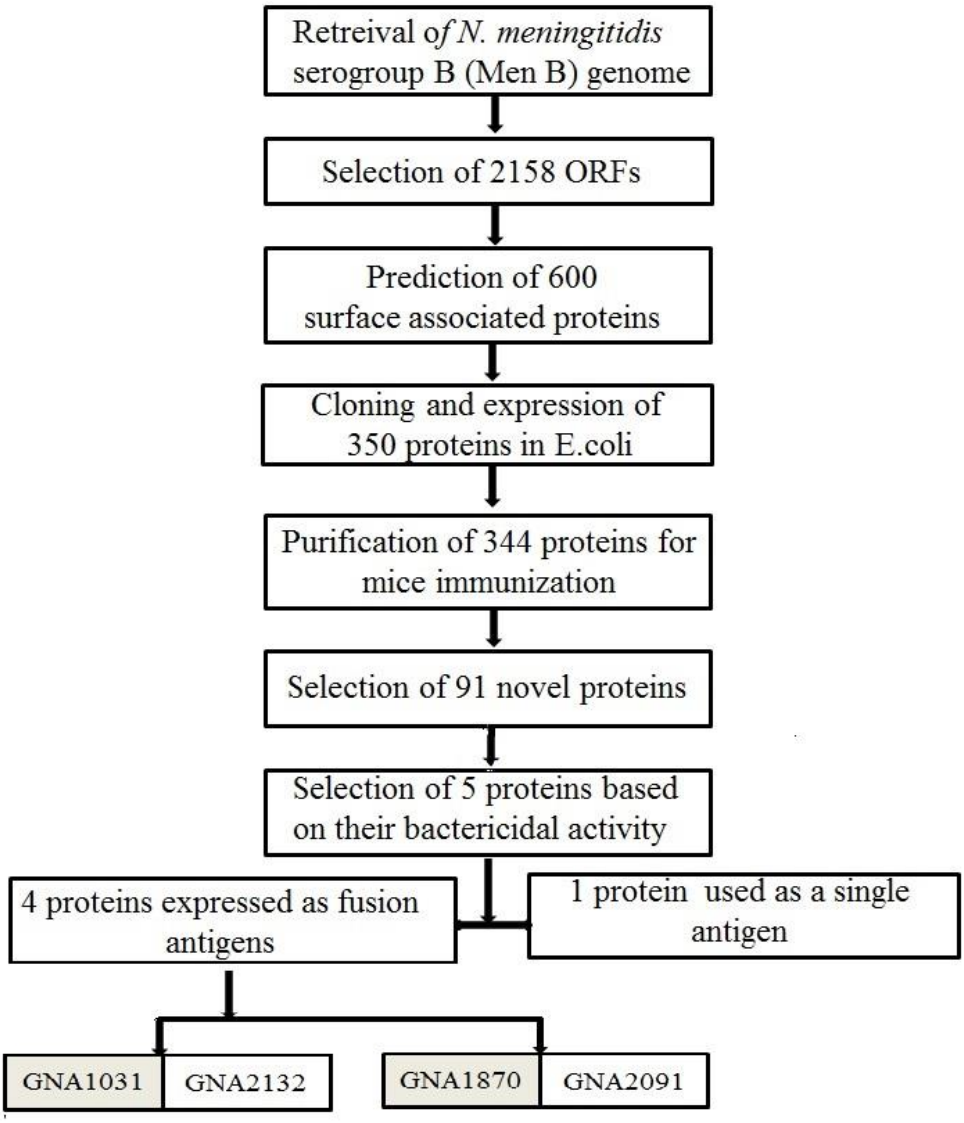
Figure 2: Screening process for MenB vaccine



From the completely sequenced MenB genome, 600 novel genes which code for surface exposed or exported proteins from 2158 were predicted ORF's. These were cultured to be expressed in *E coli* as fusions to glutatione transferase or histidine tag. From the 350 successfully expressed proteins, 344 were capable of immunizing mice. Later, 91 novel surface exposed proteins were discovered. Out of which 28 showed bactericidal activity. Based on the study, those proteins interacting with the complement system especially through H-factor and H-binding proteins, five of them were finally chosen which include GNA3132 (nmb2132), GNA1030 (nmb1030), GNA2091 (nmb2091), GNA1870 (nmb1870) and NadA (nmb1994). These 5 were used in the vaccine preparation in the form of 3 recombinant proteins GNA2132, GNA1030 as one fusion protein and GNA2010, GNA1870 as other, while NadA was a single antigen.

RV FOR TUBERCULOSIS

The causative agent of Tuberculosis is *Mycobacterium tuberculosis.* It mainly affects gastrointestinal tract, oesophagus, lungs, stomach and duodenum. The screening process resulted in the shortlisting of 3 sequences with the least sequence identity (**Table 2**) [6].

Using EMBOSS Antigenic tool, the antigenic determinants with least identity and least E value were chosen. Out of these the greatest LCV value was considered for further experimentation. The resulting antigenic determinant "HRRAPL" was docked with energy minimized MHC I molecule (206.3578 kcal/mol) and the docking energy was found to be 55.8876 kcal/mol. This analysis has proved this particular epitope to be the most potent vaccine candidate for tuberculosis. [6]

RV FOR MALARIA

Malaria, a very threatening disease is caused by *Plasmodium vivax, Plasmodium falciparum* and *Plasmodium yoelli*. The vaccines against malaria are under several trials for malarial eradication by employing conventional methods. The current vaccines for multiple stages of malaria are under clinical trials. [7]

With the availability of whole genome sequence of the parasites, RV approach has been applied to bring out an effective vaccine against the disease. Rupanjali Chaudhuri et al., developed MalVac (Database of Malarial Vaccine Candidates) database which encompasses all the details of possible malarial vaccine candidates.

The database contains 332 potential vaccine candidates. The predicted vaccine candidates by MAAP tool were further analysed by 20 publically available algorithms which includes MAAP, BLASTCLUST, TMHMM Server v. 2.0, BetaWrap, TargetP1.1, SignalP 3.0, BlastP, Antigenic, Conserved Domain Database, ABCPred, BcePred, Discotope 1.1, CEP, NetMHC 2.2, MHCPred 2.0, Bimas, Propred, AlgPred, Allermatch and WebAllergen. The database released in 2008 provides information on the vaccine candidates and immune responses in terms of orthologs, paralogs, betawraps, localization, transmembrane spanning regions, signal peptides, conserved domains, similarity to human reference proteins, T-cell epitopes, B-cell epitopes, discotopes, and allergen predictions which are required to take the vaccine development to a next stage. [7]

RV FOR LEISHMANIASIS

Leishmaniasis is caused by pathogenic species involving *Leishmania major, Leishmania donovani and Leishmania infantum.* The disease is classified into cutaneous leishmaniasis, mucocutaneous leishmaniasis and visceral leishmaniasis (VL) which can occur either separately or in certain cases coexist. Each species can cause the syndrome to various extents in different individuals [8]. The existing chemotherapeutic treatments are costly, toxic and the species develop resistance towards the drug [8]. Due to virulence, genetic differences between the species and coexistence among different forms of the disease the development of an anti-leishmanial vaccine remained difficult. But with the availability of whole genome sequence causative organisms, RV approach has become feasible and helped in the identification of novel vaccine targets. Numerous researchers have worked to identify vaccine candidates of various species.

Lijo John *et al.,* in their work, started with genome study of both *L. major* and *L. infantum* and identified all the homologous proteins from both the species using BLAST. The sub-cellular localization was traced using PSORT and extra cellular proteins in the plasma membrane were screened. These proteins were then analyzed using TMHMM and the proteins with transmembrane helices were screened. The proteins having multiple transmembrane sites were removed after a report where considering such proteins failed to clone in case of MenB vaccine. Non-homologous proteins to mouse and humans were screened and considered further to avoid any auto-immune response. Once these proteins were selected, algorithms like BIMAS, SYFPEITHI and ProPred1 were used to identify MHC class I binding epitopes and MHC class II binding epitopes were selected using ProPred. BLAST was used to remove all the MHC class I and II peptides common to both humans and mouse. The sequences with no similarity were chosen for the further studies.[8]

Juliane Schroeder *et al.,* in 2011 carried out a study to identify vaccine candidates in *L.donovani* causing dangerous form of VL. Genomic and proteomic approach involving various bioinformatics approaches were used to initiate the vaccine development process. Subcellular localization, sequence conservation across the parasite species and lack of homology to vaccine target organism were studied by former approach. The latter was used for selection based on protein expression in the amastigote form, relative protein abundance. After analyzing the sequences with 30 different algorithms to find MHC binding epitopes, the results were further studied using a non-linear predictor NetMHC (which is based on Artificial Neural Network). [9]

RV FOR HERPES

Herpes viruses belong to the herpesviridae family, a family of DNA viruses. All herpes viruses are known to be species specific. There are eight members in the human herpesviruses family namely, Herpes simplex virus (HSV) type 1 and 2 (HSV-1 and HSV-2), varicella zoster virus (VZV; HHV-3), Epstein-Barr virus (EBV; HHV-4), human cytomegalovirus (CMV; HHV-5), human herpesvirus-6 and -7 (HHV-6 and HHV-7), and Kaposi's

sarcoma associated herpesvirus (KSHV; HHV-8) which is involved in the pathogenicity in humans. The available development mechanisms for a vaccine against HSV are hindered due to the complicated mechanisms of the herpes virus which includes ccomplexity of the virus replication cycle, sophisticated immune-evasion strategies of HSV-1 and HSV-2 and 84 protein candidates encoded by large and complex herpes genome [10].

In the study carried out by Zuoshuang Xiang *et al.,* 52 herpes viral genomes were selected from NCBI and were divided into 2 classes: Human Herpes viruses (HHV) and other animal related herpes viruses. HSV-1 including strain 17, strain F and strain H129, one HSV-2 genome and 8 HHV were classified. The HSV-1 strain 17 was considered as the seed genome for vaccine design using Vaxign. All the 77 proteins of HSV strain-17 was found to be conserved in the other two strains of HSV-1 protein. Envelope glycoproteins gJ and gG were restricted only to HSV-1, which differentiated HSV-1 from all other HHV's. 7 of 77 HSV-1 genes were absent in 40 non-human herpesvirus which differentiate HHVs and non-human herpesvirus. These 7 includes envelope glycoproteins gJ and gG, neurovirulent proteins NP_044661.1 and NP_044600.1, transporter associated with antigen presentation (TAP) inhibitor ICP47, tegument protein US11 and membrane protein UL56. Only 19 of the above 77 proteins were conserved within the HHV and only UL26.5 had an adhesin probability of 0.675 which is more than the threshold (0.51). [11]

DISCUSSION

With the increasing number of deaths due to bacterial pathogens discovery of vaccines are of adverse need and reverse vaccinology technology can bridge the gaps in the vaccine development. The success of this technique depends on the availability of whole genome sequences for majority of bacterial species. Diverse bioinformatics approach has been attributed for the analysis of various surface-exposed proteins and transmembrane proteins. This process is important in terms of its cost-effectiveness and in reduction of the time taken to produce the vaccine. The RV focuses on strain coverage which is a shortcoming in conventional vaccinology. Considering the population and the economy of India, RV could be a boon in the long run as this vaccine would prevent the occurrence of epidemics among the Indian population. Due to the lesser cost in the development of vaccines through RV, it might be affordable by a larger section of Indian masses. Government subsidies and large-scale immunisation programs could be implemented to bring out a larger impact. Despite having a disadvantage that RV can be only implemented for protein vaccine candidates, RV shall continue to remain a hope to millions of people against a huge number of deadly pathogenic diseases.

CONCLUSION

Many acute infections can be controlled by vaccination but persistent infections often posses a harmful threat. The vaccine development is hindered by the emergence of drug resistant strains and cost and duration. With the advancement in the whole genome sequencing methods and availability of genome sequences of many pathogenic strains reverse vaccinology approach can be used for the development of vaccines. Reverse vaccinology an emerging and revolutionary vaccine development approach can be implemented to combat the growing bacterial diseases among various economic groups. RV has paved a great way to create one revolution in the near future, in developing much effective vaccines in terms of cost, strain coverage and reduces the time in developing the vaccine. Although now RV is not successful among viral diseases, in the future it definitely has high potential and thus the present problems can be resolved.

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REFERENCES

[1] F. Zepp, Vaccine, 2010, 28, Supplement 3, C14–C24.
[2] S. Vivona, J. L. Gardy, S. Ramachandran, F. S. L. Brinkman, G. P. S. Raghava, D. R. Flower and F. Filippini, Trends Biotechnol., 2008, 26, 190–200.
[3] D. Serruto, L. Serino, V. Masignani and M. Pizza, Vaccine, 2009, 27, 3245–3250.
[4] M. Comanducci, S. Bambini, B. Brunelli, J. Adu-Bobie, B. Aricò, B. Capecchi, M. M. Giuliani, V. Masignani, L. Santini, S. Savino, D. M. Granoff, D. A. Caugant, M. Pizza, R. Rappuoli and M. Mora, J. Exp. Med., 2002, 195, 1445–1454.

[5] P. Kashikar and C. Dipke, Journal of Advance d Bioinformatics Applications and Research, 2012, 3, 262–266.
[6] A.S. Sriranjini, Bioinfo vaccine sciences, 2011, 1, 01-06
[7] R. Chaudhuri, S. Ahmed, F. A. Ansari, H. V. Singh and S. Ramachandran, Malaria Journal, 2008, 7, 184.
[8] L. John, G. J. John and T. Kholia, Appl. Biochem. Biotechnol., 2012, 167, 1340–1350.
[9] J. Schroeder and T. Aebischer, Hum Vaccin, 2011, 7 Suppl, 10–15.
[10] G. Dasgupta, A. A. Chentoufi, A. B. Nesburn, S. L. Wechsler and L. BenMohamed, Expert Rev Vaccines, 2009, 8, 1023–1035.
[11] Z. Xiang and Y. He, BMC Bioinformatics, 2013, 14 Suppl 4, S2.
[12] A. R. Gorringe and R. Pajón, Hum Vaccin Immunother, 2012, 8, 174–183.
[13] D. Serruto, J. Adu-Bobie, B. Capecchi, R. Rappuoli, M. Pizza and V. Masignani, J. Biotechnol., 2004, 113, 15–32.
[14] J. Adu-Bobie, B. Capecchi, D. Serruto, R. Rappuoli and M. Pizza, Vaccine, 2003, 21, 605–610.
[15] M. Pizza, V. Scarlato, V. Masignani, M. M. Giuliani, B. Aricò, M. Comanducci, G. T. Jennings, L. Baldi, E. Bartolini, B. Capecchi, C. L. Galeotti, E. Luzzi, R. Manetti, E. Marchetti, M. Mora, S. Nuti, G. Ratti, L. Santini, S. Savino, M. Scarselli, E. Storni, P. Zuo, M. Broeker, E. Hundt, B. Knapp, E. Blair, T. Mason, H. Tettelin, D. W. Hood, A. C. Jeffries, N. J. Saunders, D. M. Granoff, J. C. Venter, E. R. Moxon, G. Grandi and R. Rappuoli, Science, 2000, 287, 1816–1820.
[16] B. C. Ross, L. Czajkowski, D. Hocking, M. Margetts, E. Webb, L. Rothel, M. Patterson, C. Agius, S. Camuglia, E. Reynolds, T. Littlejohn, B. Gaeta, A. Ng, E. S. Kuczek, J. S. Mattick, D. Gearing and I. G. Barr, Vaccine, 2001, 19, 4135–4142.
[17] R. A. M. Jong and W. A. van der Reijden, Expert Rev Vaccines, 2010, 9, 193–208.
[18] R. Rappuoli and A. Aderem, Nature, 2011, 473, 463–469.
[19] W. Gan, G. Zhao, H. Xu, W. Wu, W. Du, J. Huang, X. Yu and X. Hu, Parasitol. Res., 2010, 106, 873–882.
[20] A. J. McCarthy and J. A. Lindsay, BMC Microbiol., 2010, 10, 173.
[21] C. Giefing, A. L. Meinke, M. Hanner, T. Henics, M. D. Bui, D. Gelbmann, U. Lundberg, B. M. Senn, M. Schunn, A. Habel, B. Henriques-Normark, A. Ortqvist, M. Kalin, A. von Gabain and E. Nagy, J. Exp. Med., 2008, 205, 117–131.
[22] C. Morsczeck, T. Prokhorova, J. Sigh, M. Pfeiffer, M. Bille-Nielsen, J. Petersen, A. Boysen, T. Kofoed, N. Frimodt-Møller, P. Nyborg-Nielsen and P. Schrotz-King, Clinical Microbiology and Infection, 2008, 14, 74–81.
[23] S. Montigiani, F. Falugi, M. Scarselli, O. Finco, R. Petracca, G. Galli, M. Mariani, R. Manetti, M. Agnusdei, R. Cevenini, M. Donati, R. Nogarotto, N. Norais, I. Garaguso, S. Nuti, G. Saletti, D. Rosa, G. Ratti and G. Grandi, Infect. Immun., 2002, 70, 368–379.
[24] O. Finco, A. Bonci, M. Agnusdei, M. Scarselli, R. Petracca, N. Norais, G. Ferrari, I. Garaguso, M. Donati, V. Sambri, R. Cevenini, G. Ratti and G. Grandi, Vaccine, 2005, 23, 1178–1188.
[25] Y. Li, S. K. Ahluwalia, A. Borovkov, A. Loskutov, C. Wang, D. Gao, A. Poudel, K. F. Sykes and B. Kaltenboeck, Vaccine, 2010, 28, 1598–1605.
[26] T. Hasegawa, M. Minami, A. Okamoto, I. Tatsuno, M. Isaka and M. Ohta, Microbiology (Reading, Engl.), 2010, 156, 184–190.